DERIVATIZATION OF RIBONUCLEASE A: AN HPLC AND MASS SPECTROMETRIC ANALYSIS OF MODIFICATION BY CHEMICAL AND MUTAGENIC METHODS

By

TRISHA JOY MÉNDEZ

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1999

AKNOWLEDGEMENTS

My time spent at the University of Florida will always represent for me a rather remarkable time in my life. Many people have contributed to making the time valuable, not only with regard to the well-rounded education I have received but also the memories that have been created. I wish to address those who have put a great deal of effort into my endeavors.

No education is possible without an environment in which to flourish. Therefore, I would like to thank the establishment, the University of Florida, the Graduate School, College of Liberal Arts and Sciences and the Department of Chemistry for allowing me to join and learn within this institution. Specifically I acknowledge the stipend and tuition waivers I received as well as travel funds, and I am grateful for this financial support.

I wish to thank all those who have been my colleagues in one capacity or another. I shall start with those have worked with me day in and day out, the Richardson research group past and present. It has been a pleasure to work with and get to know Ken Weakley, Celeste Regino, Deon Bennett, Linda Nichols, Huirong Yao, Cheng Xu, Jerrold Miller and Matt Ryan. Each person has enlivened and added an important dimension to the friendly atmosphere of the Richardson research labs. I will miss these friends. I specifically acknowledge Celeste Regino for the synthesis of the DPA ligands and for aid in the coupling reactions, and I am grateful to Linda Nichols for the acquisition of all ESI/FTICR-MS data presented here.

I wish to thank those people in the mass spectrometry services lab who have become respected colleagues: Dr. David Powell and Dr. Jodie Johnson. I thank Dr.

Powell for the acquisition of the MALDI/TOF-MS data presented in this dissertation, his much appreciated editing skills for the preparation of this manuscript as well as his financial support. I am grateful to Jodie for the acquisition of all HPLC/ESI/QIT-MS data presented. I also thank Jodie for his great ideas, smiling face, quality of character and ability to listen that have made our friendship of highest value to me. I also must thank Dr. Nancy Denslow for the use of the MALDI/TOF-MS in the ICBR Protein Core and for her advice in protein chemistry.

I want to thank the people in the Benner group who have contributed to my efforts in different ways. Lee Raley has performed a significant amount of work for the creation of the mutant RNase A and taught me what I know about protein expression. Stefan Lutz has been a colleague beyond compare from the very beginning. From classes to qualifying exams to practicing for presentations, it has been my good fortune to have Stefan as my classmate and friend. Dr. Steven Benner has been generous in his sharing of equipment for my research and enlightening as a teacher, one I hope to never forget.

I cannot forget the people who make the bureaucracy of school much easier to navigate: all those in the chemistry fiscal office, the graduate coordinator's office and the inorganic divisional offices, especially Amanda Garrigues and Maribel Lisk.

My time at UF would never have been as enjoyable as it was if not for the friendships of many, including Perla Sklar, Julia Rusak, Linda Nichols, and most importantly Jennifer Tonzello. These women will be lifelong friends.

My deep appreciation goes to my research advisor, Professor David Richardson. Dave has helped me become a better researcher, improve my writing skills and learn how to thicken my skin, all skills that will improve my professional future. The areas in which and the sheer volume of information I learned from Dave cannot be described here. Let it be sufficient that I say I am truly grateful for his efforts as an advisor.

Finally, my heart felt thanks goes to my immediate family, whose love for me has made possible everything good in my life. My mother, Lora, has been my biggest cheerleader and most helpful friend. Her courage and work ethic have been the best example of a woman to be emulated. My son, Alexander, has made me a more efficient student, but more importantly his presence has taught me gratitude for things most important in life. My husband, Edras, has been my partner and friend for longer than I can remember. His contributions to my life seem inseparable from my own, and the work presented here is just one of many endeavors that would not be possible without his unconditional love. Most importantly I thank the good Lord for His abundant grace.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS ii

ABSTRACT vii

CHAPTERS

1 INTRODUCTION 1

Chemical Modification of Proteins 1
Electrospray Mass Spectrometry 12
ESI-MS Applications to Protein Characterization 18
ESI-MS for Analysis of Chemical Modifications and Bioconjugation in Proteins 21

2 CHEMICAL MODIFICATION OF RIBONUCLEASE A 30

Introduction 30
Experimental Procedures 36
Results and Discussion 41
Conclusions 66

3 THE MAPPING OF SMPT-MODIFIED RNASE A I: PROTEOLYTIC DIGESTION WITH TRYPSIN AND ESI/FTICR-MS ANALYSIS 69

Introduction 69
Experimental Procedures 74
Results and Discussion 77
Conclusions 87

4 THE MAPPING OF SMPT-MODIFIED RNASE A II: PROTEOLYTIC DIGESTION WITH SUBTILISIN AND HPLC/ESI/QIT-MS AND MS^n ANALYSIS 88

Introduction 88
Experimental Procedures 91
Results and Discussion 93
Conclusions 113

5 BIOCONJUGATION OF RNASE A TO A SYNTHETIC COFACTOR: PRELIMINARY WORK 114

Introduction 114
Experimental Procedures 119
Results and Discussion 123
Conclusions 129

6 GENERAL CONCLUSIONS 132

APPENDIX 138

LIST OF REFERENCES 144

BIOGRAPHICAL SKETCH 160

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DERIVATIZATION OF RIBONUCLEASE A: AN HPLC AND MASS SPECTROMETRIC ANALYSIS OF MODIFICATION BY CHEMICAL AND MUTAGENIC METHODS

By

Trisha Joy Méndez

December 1999

Chairman: Professor David E. Richardson
Major Department: Chemistry

Chemical modification of proteins is a demonstrated method for incorporating new ligands into an enzyme through the reaction of residue side chains with various chemical reagents. Reaction of an enzyme residue with a modifying reagent that incorporates a ligand of interest to the system results in potential novel chemical activity of the enzyme, creating a bioconjugate. A bioanalytical method for the development and characterization of a base protein system to be used as a bioconjugate is presented.

The modification of ribonuclease A with the heterobifunctional cross-linker, 4-succinimdyloxycarbonyl-methyl-α-[2-pyridyldithio]-toluene (SMPT), is described. RNase A has 11 sites of potential modification by the SMPT reagent. Tracking the 2-dimensional separation and proteolytic digestion of SMPT-modified RNase A with ESI/FTICR-MS and HPLC/ESI/QIT-MS demonstrates the detailed information about the number of SMPT modifications and sites of modification that can be obtained by application of these techniques. Analysis of native and modified RNase A tryptic digests by ESI/FTICR-MS resulted in the identification of the sites of modification. Semi-quantitative results of the reactivity of certain lysine residues toward the coupling reagent SMPT are possible. Two sites (lysines 1 and 37) are highly reactive, while three sites (lysines 41, 61 and 104) appear to be unreactive under the conditions used. Digestion of the modified and unmodified RNase A by subtilisin followed by examination with HPLC/ESI/QIT-MS resulted in the quantification of the propensity of subtilisin to cleave RNase A and SMPT-modified RNase A at various peptide linkages, which has not been previously demonstrated. HPLC/ESI/QIT-MS and MS^n enabled further investigation of modification on lysines 1 and 7, including modification at the ε- and α-amino positions on lysine 1. Coupling of singly modified RNase A to a synthetic cofactor of interest, 2,2'-dipyridylamine, is demonstrated as an example of the assembly of the bioconjugate.

The complement to chemical modification of RNase A for the development of a system for bioconjugation is site-directed mutagenesis. Preliminary work on the characterization and coupling of the mutated RNase A (F8C) using MALDI/TOF-MS is presented.

CHAPTER 1
INTRODUCTION

Chemical Modification of Proteins

The chemical modification of proteins is a useful tool for the elucidation of information most basic to enzyme chemistry, that is, the understanding of substrate binding and subsequent bimolecular catalysis. The rational design of chemical modifiers has enabled structural information about proteins and enzymes to be obtained prior to the modern techniques of X-ray crystallography and nuclear magnetic resonance (NMR) spectrometry. By introducing new chemical groups into a protein via covalent interaction, information can be learned about the chemical nature and relative reactivities of the amino acids that are present. Residues most susceptible to chemical modification are lysine and arginine at their nucleophilic ε-animo groups, tyrosine at its hydroxy group, cysteine at its sulfhydryl group or cross-linked disulfide, histidine at its imidazole group, tryptophan at its indole group, glutamic acid and aspartic acid at their carboxylic acids, the N-terminal amino, and C-terminal carboxyl groups. Due to the differences in the chemical nature of these groups, their reactivities vary with different chemical modifiers, changing pH, and buffer composition (1-3). Exploitation of these chemical differences through chemical modification has resulted in thousands of publications demonstrating structural and functional information for proteins. An overview of research examples is presented here illustrating not only the evolution of chemical modification as a standard tool for the investigation of proteins but also creative expansions to the basic chemical modifications for the accomplishment of innovative research goals.

One of the first comprehensive chemical modification studies was demonstrated in 1948, by Fraenkel-Conrat *et al.* (4). These researchers investigated the protease trypsin, its inhibitor, ovomucoid and the mode of interaction between the two through chemical modification. A series of chemical derivatizations were performed on trypsin and ovomucoid including acetylation, esterification, epoxidation, reduction, coupling with diazobenzenesulfonic acid, iodination, and so on. Such modifications resulted in the determination that trypsin interacts with a substrate through groups other than its amino groups but combines through its amino groups with the acid groups of the inhibitor, ovomucoid. A stabilization role was assigned to the other important residues in trypsin.

Fraenkel-Conrat *et al.* (5) used chemical modification again in 1950 to probe the iron-binding capability of conalbumin, an enzyme found in egg whites. It was reported that all chemical modifications performed in this research, including acetylation, iodination or coupling of phenolic acid and imidazole groups, substitution of amide of guanidyl groups and disulfide reduction, resulted in inactivation of iron-binding capability, demonstrating the complexity of the interactions required for metal-binding. Analytical techniques available at that time did not allow further information to be extracted from the chemical modification results, despite the understanding of the chemistry involved in the modifications.

In 1962, a general method for covalent modification of active site residues was presented by Wofsy *et al.*(6). Even though the specific example used to demonstrate the theory was an antibody, its direct application to enzymes was clear. The method, termed affinity labeling, made use of a modification reagent that is designed to have an affinity for a specific site on the protein. Once bound by non-covalent interactions, the reagent and protein undergo a chemical reaction to covalently bind the reagent or part of the reagent to the protein in the selected site. However, it was noted that "...conclusive proof that labeling of the active site had occurred could only be achieved through the accumulated weight of a great many different types of experiments." This statement demonstrated the need for improved biological and analytical methods to realize the potential of chemical modification reagents to shed light on these complex biological systems.

The development of Edman sequencing became a revolutionary method for the determination of primary sequences of proteins and protein fragments and also lent itself to the determination of the residues that had been chemically modified in the protein of interest (7-10). With this technique well established, the usefulness of chemical modification as a method for probing proteins increased. Such usefulness was documented in 1972 in *Methods in Enzymology*, a detailed review of chemical modification reagents to date, where an evaluation of the modification reagents, the biochemical reactions in which they are involved, and the information which could be obtained from these reactions was presented (11,12). The aforementioned advances facilitated the identification of residues involved in binding and catalysis and saw the beginning of chemical modification of proteins as a key tool in the expansion of knowledge and the advancement of research possibilities in protein and enzyme chemistry.

The affinity labeling theory presented by Wofsy in 1962 (6) was supported by the expansion of chemical modification possibilities. An example of research with affinity labeling was demonstrated by Means and Feeney in 1971 (13) where bovine pancreatic ribonuclease (RNase) was complexed to the reagent pyridoxyl phosphate (PLP). The PLP interacts with the phosphate-binding site upon reduction with sodium borohydride (Figure 1-1). The loss of activity upon binding of PLP revealed structural and activity information about substrate binding to RNase.

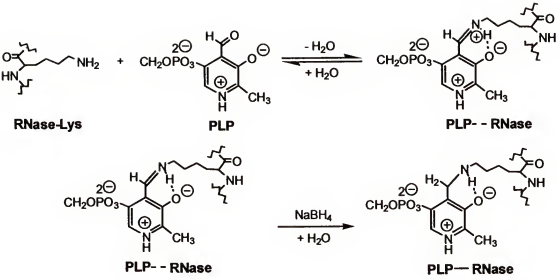


Figure 1-1. Affinity labeling of RNase at lysine residues by pyridoxal 5' phosphate (PLP). The PLP phosphate group interacts with the binding domain in RNase while the RNase lysine residues form Schiff base interactions with the PLP aldehyde functionality. Reduction of the Schiff base linkage by sodium borohydride ($NaBH_4$) results in covalent attachment of PLP to RNase lysine residues.

Once affinity labels had become a standard for protein structure-function studies, increasing functionality was added to the affinity label. Control over the chemical reactivity of the affinity label was demonstrated by Jelenc *et al.* (14) with the development of photo-reagents, 4-nitrophenyl ethers, as affinity labels and protein cross-linkers. The 4-nitrophenyl ethers react with amino groups at physiological pH only upon irradiation with 366 nm light (Figure 1-2). The result was a substituted nitrophenyl amine in high yield compared to its acetyl and alkyl amine counterparts, which reacted with decreased specificity with the protein and sometimes with reaction buffers. Higher specificity due to the photosensitive nature of the 4-nitrophenyl esters gave high yield of modified product, and increased the possibility of attaching functionality to the protein to aid in characterization.

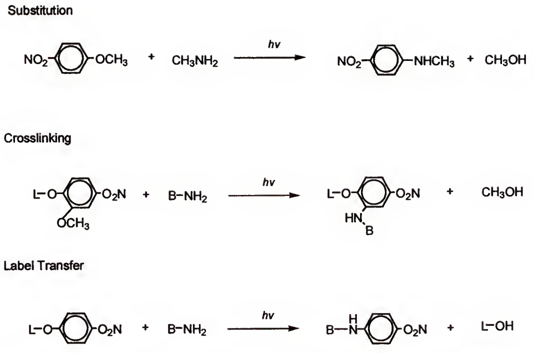


Figure 1-2. Reactions of 4-nitrophenyl ether for as a cross-linker or affinity label.
Substitution: Irradiation of 4-nitrophenyl ethers in the presence of primary amines results in substitution of the ether linkage with an amine.
Cross-linking: Nitrophenyl ether is attached to a ligand or macromolecule, L, and in the presence of a biological molecule possessing a primary amine, B, a cross-link is formed when the system is irradiated with 366 nm light.
Label Transfer: Substitution occurs in biological systems, as in the first reaction, resulting in the transfer of the nitrophenyl ether to the biological molecule, B, from the nitrophenyl ligand, L. Reaction schemes reproduced from Jelenc *et al.* (14).

The applicability of using a chemical modifier as an affinity label expanded to include determination of binding interactions and catalytic mechanisms. An example of such a study was presented by Klein *et al.* (15) with the investigation of the binding of the natural adenosine triphosphatase (ATPase) inhibitor (IF_1) with beef mitochondrial ATPase. The inhibitor was chemically modified with phenyl (^{14}C) isothiocyanate labels (^{14}C PITC) (Figure 1-3). Study of the interactions between the ATPase and the labeled inhibitor resulted in data regarding binding affinity, stoichiometry, and localization of binding sites in ATPase. Such information was used to further the understanding of the regulatory functions of IF_1 in oxidative phosphorylation. Also used in this study was a chemical modifier as a cross-linker that covalently bound the ATPase and IF$_1$ for analysis by gel electrophoresis.

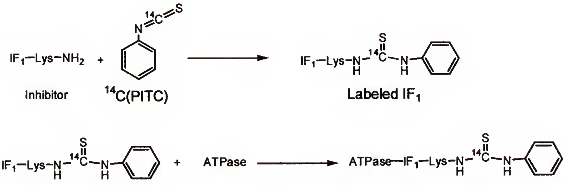
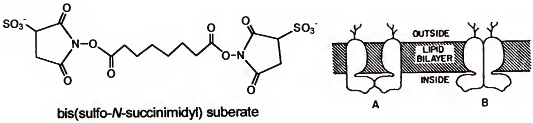


Figure 1-3. Beef mitochondrial ATPase inhibitor, IF$_1$, was labeled with phenyl (^{14}C) isothiocyanate (^{14} PITC) as in Scheme 1. The labeled IF$_1$ was then complexed to ATPase, as in Scheme 2, to study binding affinity, stoichiometry and to aid in localization of binding sites in ATPase.

PITC and its polar analog *p*-sulfophenylisothiocyanate (*p*-sulfoPITC) were later used as chemical modification reagents by Genchi *et al.* (16) to probe the molecular mechanism of the phosphate transport protein of bovine heart mitochondria. Labeling the lysine residues with the affinity reagents showed that previously investigated amino groups, important for transport activity, were topologically related to the sulfhydryl groups already known to be essential for phosphate transport (17). It was demonstrated that the modification of lysine residues altered the protein's chemical characteristics whether modification occurred on surface lysine residues or those buried in the hydrophobic region of the phosphate transporter.

As the structural information that could be obtained from chemical modification of proteins increased, the protein systems upon which researchers focused turned to more challenging subjects such as membrane proteins. Because the membrane protein is deeply imbedded in the cellular lipid bilayer, manipulation of the protein could not be accomplished with the same methods as those used for soluble proteins.

Staros (18) presented a series of chemical modifiers used as cross-linking reagents that were developed specifically for transmembrane proteins. Membrane-impermeant cross-linking enabled protein subunits to be cross-linked prior to disruption of the lipid bilayer (Figure 1-4). Analysis of the linked subunits probed questions such as the identity of the fundamental oligomeric unit and whether the intersubunit interactions exist only between the cytoplasmic domains or also between transmembrane domains. The cross-linking, bifunctional reagents presented by Staros enable the investigation of tertiary and quaternary structures and interactions among membrane proteins and extracellular structures. Such investigations would not be possible without the rational design of chemical modifiers, whether monofunctional for labeling or bifunctional for labeling/cross-linking experiments.



bis(sulfo-*N*-succinimidyl) suberate

Figure 1-4. An example of a membrane-impermeant cross-linker, bis(sulfo-*N*-succinimidyl) suberate. The reagent was used to cross-link membrane spanning proteins. An example of this cross-linking is shown for a transmembrane protein. Cross-linking of the protein subunits with the membrane-impermeant reagent occurred "outside" in the extracellular environment. Subsequent lysing of the transmembrane domain followed by oxidation resulted in the formation of intersubunit disulfide bonds on the "inside" cytoplasmic domain. Cross-linkage between both extracellular and cytoplasmic domains indicated the transmembrane domains were juxtaposed, as in B. Linkage solely between cytoplasmic domains would have indicated the transmembrane domains were not associated in the extracellular environment, as in A. Pictures reproduced from Staros (18).

A further use of chemical modification has been the elucidation of immunological information. Ebina *et al.* (19) used a series of chemical modifications as protecting groups to localize a further cross-linking modification that coupled the protein bovine pancreatic trypsin inhibitor (BPTI) to an immunogenic peptide (Figure 1-5).

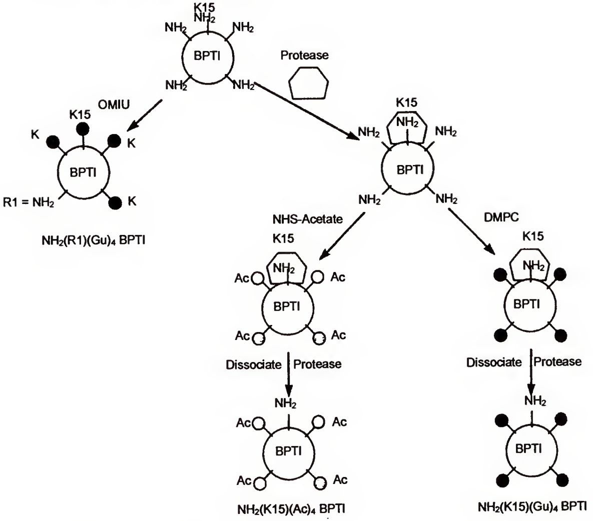
Once coupled, the BPTI and immunogenic peptide could be studied by NMR to investigate the relationship between the BPTI/immunogenic peptide conformation and the peptide's ability to induce an immune response. Such examples of the use of chemical modification illustrate its position as a standard tool in the changing field of biochemistry.

The ability to study the kinetic activity of an enzyme system has also become a product of creative affinity labeling. Typical information obtained through kinetic studies of an enzyme with an affinity label was shown in the investigation by Chang *et al.* (20) in the modification of human placental alkaline phosphatase with the affinity label periodate-oxidized 1, N^6-ethenoadenosine monophosphate (εAMP-dial) and its more stable derivative, 4',5'-anhydro-1, N^6 ethenoadenosine dialdehyde acetal (AεAdo-dial) (Figure 1-6). Interest in this system was high due to the protein's role as a plasma membrane phosphate transporter and the affinity label's reported role in anti-tumor activities (21-23). Kinetic data obtained from monitoring the fluorescence of the labels was used to gain structural information regarding the uncharacterized active center. A hydrophobic binding pocket was postulated as well as a binding site lysine.

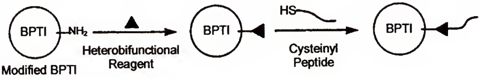
An important part of chemical modification studies has become the knowledge of the residue that has been modified. Early experiments looked for placement in the three dimensional structure of an enzyme, but the automation of Edman sequencing now allows for rapid sequencing of large, proteolytically digested proteins and the identification of the modification site. Peptide mapping of proteins (24) has been

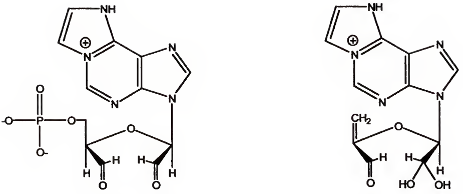
Figure 1-5. Schematic representation of the coupling of bovine pancreatic trypsin inhibitor (BPTI) to an immunogenic peptide. A) Three methods for the preparation of BPTI with a modifiable lysine residue localized to one site. The first method involves the reaction of BPTI with *O*-methyl isourea (OMIU) that blocks all lysine residues except the amino-terminal amino group. The other method involves the interaction of BPTI with a protease that interacts with a specific lysine residue, lys-15. While complexed to the protease, the BPTI is chemically modified at all other amino groups by either acetylation of guanidation. Dissociation of the modified BPTI from the protease results in a single free lysine residue. B) The modified BPTI is then coupled to a heterobifunctional reagent followed by linkage to the immunogenic peptide. Reaction schematics reproduced from Ebina *et al.* (19).

A. Formation of Modified BPTI's



B. Coupling of Modified BPTI's



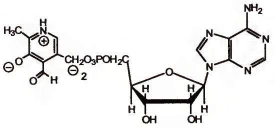


εAMP-dial

AεAdo-dial

Figure 1-6. Affinity reagents used for the modification of human placental alkaline phosphatase. A) Periodate oxidized 1, N^6-ethenoadenosine monophosphate (εAMP-dial). B) β-elimination product of εAMP-dial, 4',5'-anhydro-1, N^6-ethenoadenosine dialdehyde acetal (AεAdo-dial). Structures reproduced from Chang *et al.* (20).

successfully applied in determination of the site of modification. An example of peptide mapping of a chemically modified enzyme was demonstrated by LaDine *et al.* (25) in their 1991 investigation of yeast-3-phosphoglycerate kinase. An in-depth kinetic study of this enzyme with a specific inhibitor, [^{3}H]pyridoxyl 5'-diphospho-5'-adenosine (PLP-AMP) (Figure 1-7) was performed, followed by peptide mapping of the protein once the inhibitor had been covalently attached by reduction with sodium borohydride.



[^{3}H] PLP-AMP

Figure 1-7. Structure of [^{3}H] pyridoxal 5'-diphospho-5'-adenosine (PLP-AMP). PLP-AMP is a specific inhibitor for yeast-3-phosphoglycerate kinase. Once PLP-AMP binds to yeast-3-phosphoglycerate kinase, reduction with $NaBH_4$ results in covalent attachment of the label to the protein.

High performance liquid chromatography (HPLC) was used to separate the peptides resulting from proteolytic digestion by endopoteinase Lys-C. From these experiments, not only was information learned about the kinetic interactions of the inhibitor PLP-AMP with the kinase, but information was also gained regarding the conformational changes of the enzyme in solution during catalysis. It was demonstrated that Lys-131 undergoes substantial movement during a ligand-induced conformational change that had been shown to occur during formation of the ternary complex, thereby positioning the positively charged residue near a negatively charged substrate. Such a study illuminated the tight binding of nucleotides by bi-lobial kinases.

Covalent modification of proteins with specific derivatizing agents has become a standard procedure for probing topology of enzyme binding sites, gross and local conformational changes, subunit interactions, characteristics of substrate binding and the reactivity of amino acid side chains. Such investigations would not be possible without the proper analytical tools for analysis including ultraviolet-visible (UV-vis) spectroscopy, fluorescence spectroscopy, NMR, rapid Edman sequencing and HPLC. However, the analytical technique that has increasingly lent itself to characterization of chemical modifications on proteins is mass spectrometry.

Electrospray Mass Spectrometry

The growth in use of mass spectrometry (MS) as an analytical tool has enabled biochemists to examine proteins in a fashion unlike earlier techniques. Mass spectrometry is a technique that allows the mass-to-charge ratio (m/z) of an ion to be determined in the gas phase. Introduction of the molecule of interest (analyte) into the mass spectrometer is accomplished through an ionization source. Ionization of the analyte as well as transformation of the ion to the gas phase has been the focus of the development of various instruments and techniques for introduction into the mass spectrometer (26-28). More recent ionization methods such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have overcome problems that originally kept mass spectrometry from being used with biomolecules.

Original ionization methods did not allow biological molecules to be vaporized without extensive decomposition. These "hard" ionization methods used gas-phase encounters of the molecule with either electrons as in electron impact or electron ionization, with photons as in photoionization, or with other ions (reagent gas) as in chemical ionization (27,28). While these ionization techniques were appropriate for small volatile molecules, they were not useful for larger, sensitive, and less volatile biomolecules that began to thermally decompose prior to ionization. The development of "soft" ionization methods has enabled mass spectrometry to be useful in biomolecular analysis. "Soft" ionization is based on the rapid dispensal of energy on a surface over which the species to be analyzed has been dispersed. Beuhler *et al.* (29) proposed that rapid energy input may result in vaporization prior to decomposition of the molecule. Since this proposal in 1974, many "soft" ionization methods have been developed (30) including plasma desorption (PD) (31), fast atom bombardment (FAB)(32,33), laser desorption (LD)(27), field desorption (FD) (34), electrohydrodynamic (EH) ionization (35,36), and spray ionization (27,37). Of these various techniques, the spray ionization technique of electrospray and the LD technique of MALDI have become widely used ionization methods due to their ability to produce multiple intact ions of large biomolecules for the determination of their relative molecular masses. Species > 100 kDa have been observed.

Extensive work has been done in probing the mechanisms of action and possible results of these two techniques. MALDI has generally been explained by photoionization followed by ion-molecule reactions that result in the production of ions in the plume of ejected material (38). Analysis of the ions is frequently accomplished by a time-of-flight

(TOF) mass analyzer. The resulting spectra generally contain peaks from singly protonated intact molecules with decreasing amounts of doubly and triply protonated intact molecules. Such an accepted and widely used technique has been the subject of many investigations and reviews (26,38-41). In comparison to MALDI, ESI produces multiply charged molecules from solution arising from the attachment of protons or positively charged salts in positive ion formation, or from the removal of protons in negative ion formation. ESI spectra contain complex charged-state distributions that are the result of the differing mechanism of ionization from that of MALDI (37,38). Original descriptions of electrospray were provided by Dole *et al.* (42,43) who originally conceived of the idea of electrospray. The development of this technique as an interface for the combination of liquid chromatography and mass spectrometry includes extensive work over the course of 20 years, and is reviewed by Loo (39).

The mechanism of electrospray ionization can be divided into two main steps described by Bruins (44): 1) nebulization of a sample solution into electrically charged droplets, 2) liberation of ions from droplets. Transportation of the ions from the atmospheric pressure source region into the vacuum and mass analyzer for detection then follows. Bruins described nebulization as a combination of the formation of a fine mist of droplets from a needle tip and charging of those droplets. This nebulization occurs when a liquid sample is fed through a needle and a high electric field is applied at the needle tip. The electric field pulls the positive charge in the sample toward a liquid front. Electrostatic repulsion and the surface tension at the tip then results in small electrically charged droplets leaving the surface and entering the surrounding ambient gas. High voltage that is applied to the tip results in the release of charged droplets as a spray into the voltage drop in the surrounding ambient gas. A drop in pressure inside a counter electrode results in the charged droplets being drawn into the counter electrode where droplet disintegration later occurs.

Flow rate, surface tension, and analyte concentration affect nebulization. Increases in any of these can reduce the formation of small charged droplets and result in larger droplets with reduced charge. Some modifications to the basic electrospray system to increase tolerance toward these adverse effects include dilution of the aqueous solution with an organic solvent such as methanol to reduce the surface tension and addition of a high-velocity gas flow to aid in aerosol formation with increased flow rate.

Optimization of such an electrospray system results in the formation of highly charged small droplets that are then desolvated during their flight through gas at atmospheric pressure. Heat and decreasing pressure, as well as shear forces, strip the solvent from the droplets reducing their size while the molecular temperature remains low (approximately 50°C) due to evaporation (45). The decreasing size of the droplet results in increasing charge density at the surface, deforming the droplet, and leading to "Coloumbic explosion," where the "parent" droplet explodes into "daughter" droplets or microdroplets, which are ejected from local deformation on the "parent" droplet, releasing the "daughter" droplets in a type of stream (46,47). Once a droplet has a radius of approximately 10 nm the charge density is reduced by ejection of ions from the surface leading to naked sample ions in the gas phase that are then analyzed. A more detailed explanation of the mechanism of electrospray has been presented by various authors (47-53).

According to the mathematical derivation presented by Kebarle *et al.* (46,54), the abundance of a sample ion is proportional to the charge on the droplets and the sensitivity coefficient, which is dependent on ion structure. The ESI system allows for multiple charging of the molecule and is thus directly applicable to large biomolecules because of their inherent sensitivity and high molecular mass. Increased charging of the large molecule decreases the mass-to-charge ratio (m/z) allowing detection by instruments with a limited m/z range (55). It was initially thought that sample ion signal should be proportional to sample concentration, but independent from flow-rate. Bruins questioned whether ESI is truly concentration sensitive or whether apparent concentration sensitivity is the result of opposing physical and chemical effects. Experimental results from other researchers (49,56) and those described below indicate that ESI is not truly concentration sensitive and that comparative concentration studies can only be performed on ions of similar chemical nature with the same charged state.

The applicability of ESI to biomolecular analysis is apparent in the growth in number of researchers using ESI as a primary analytical tool. There are vast examples of researchers who have used ESI-MS to determine the molecular masses of endogenous structural biopolymers such as peptides, proteins, glycoproteins, and glycolipids, as well as lower mass molecules such as fatty acids, vitamins, steroids, and nucleic acids (57). An extensive review of ESI-MS used to study noncovalent interactions in biomacromolecular complexes was presented Przybylski *et al.* (45).

The addition of liquid chromatography to ESI has also increased the number of samples examined by MS by facilitating sample preparation and introduction into the ESI source. A review of the coupling of liquid chomatography (LC) to ESI and its applications was presented by Mehlis *et al.* (58) and Dass *et al.* (59).

The characterization of proteins at the primary structural level has become a major use of ESI through peptide mapping strategies. Reduction of high molecular weight protein to smaller molecular weight peptide segments is accomplished through proteolytic digestion. Sequence analysis is then performed by tandem MS (MS^n), which is mass spectrometric selection of a parent ion followed by fragmentation of the parent ion into its daughter ions and their neutral constituents.

$$A^+ \longrightarrow B^+ + C$$

In the studies presented here, ion fragmentation was achieved through collisionally activated dissociation (CAD), also known as collision induced dissociation (CID). A thorough discussion of CID was presented by Hunt *et al.*, along with its applicability to peptide sequence analysis (60); however, a brief discussion follows. Ions that are to be fragmented by CID are mass selected, and all other ions are ejected from the trapping cell. The trapping cell contains a neutral gas such as helium or argon with which the ions of interest collide at a low collision energy (15-40 eV). The kinetic energy of the collisions is converted to vibrational energy in the ions with the parent ion's internal energy becoming so high that it fragments randomly at amide bonds along the peptide backbone. Loss of water, ammonia, and phosphoric acid also occurs. The resulting daughter ions are analyzed while neutral molecules are pumped away. Analysis of the fragmentation data enables the peptide sequence to be elucidated.

Tandem MS is a term used to describe the mass spectrometric selection of an ion followed by mass spectrometric fragmentation, usually by CID. Once a fragmentation pattern has been obtained from CID, a fragment ion can be selected and fragmented further. General mass selection and fragmentation has been termed MS^n, indicating the possibility of multiple ion isolations and fragmentations. Current MS peptide sequencing has been adapted to integrate database searching and to facilitate identification of unknown proteins from CID fragmentation data (61-63). This technique is known as peptide mass fingerprinting (64). Further reviews of peptide sequencing and mapping techniques include those by Hunt *et al.*, (41), Gelpi (57), Suizdak (26), Covey (65), Loo (39), and Dass (59).

ESI-MS Applications to Protein Characterization

Recent examples of peptide sequencing include the structural determination of post-translational modifications to proteins. Covalent modifications to proteins are an integral part of cell regulation. Discovery of the location and identities of these modifications provides information regarding such cell regulation and protein function. Post-translational modifications include glycosylation, phosphorylation, sulfonation and acetylation (39).

Proteolytic digestion of a protein with such a modification followed by ESI-MS yields data that indicates the nature of the modification. A classic example of such an experiment was reported by Hunt *et al.* (60), where class III ß-tubulin was examined for post-translational modification on an acidic region of the C-terminal end of the protein. The protein was digested with cyanogen bromide, and the peptide segments with the C-terminal end were isolated by complexation to a monoclonal antibody, TuJ1. Once separated from the antibody, the peptide sequences were subjected to ESI-MS after purification by HPLC. MS data yielded two multiply charged ion series. One series of peaks of charge $[M + H]^+$ were separated by 143 Da, indicating modification by glutamic acid. Another series, also of charge $[M + H]^+$, were separated by 80 Da and 143 Da indicating modification by phosphate and glutamic acid. CAD was performed on the peptides that were identified to have post-translational modification, and the resulting data showed the first two residues at the amino acid terminus of the chain of interest contained the phosphate and glutamic acid on residues Tyr 437 and Glu 438.

In 1992, two papers were published by Hunt *et al.* demonstrating the power of HPLC/ESI-MS/MS to solve complex cellular problems (66,67). These coupled techniques were used to identify and sequence peptide antigens bound to a major histocompatibility complex (MHC) molecules of class I. The MHC binds the peptide antigens after they have been transported into the endoplasmic recticulum from inside the cytoplasm of cells or viruses. HPLC/ESI-MS/MS was used to separate and characterize sets of these numerous antigen peptides, overcoming the drawbacks associated with the previously used techniques of Edman degradation (peptides too long for complete sequencing) and HPLC (poor separation for sub-picomole amounts of peptide). Examination of these antigen peptides using the MS method demonstrated its use in analysis of small quantities of virally infected and transformed cells as well as those associated with autoimmune diseases.

Information obtained from ESI-MS has grown from exact mass and sequence determination to structural conformation and mechanistic investigations. Tracey *et al.* reported on the structural and mechanistic aspects of the soluble dimeric ß-galactoside-binding lectin of bovine heart muscle (68). This enzyme binds carbohydrates when in the reduced state but loses this activity upon oxidation. ESI-MS was used to demonstrate the lack of post-translational modification as well as to confirm changes in the reduced and oxidized forms of the protein. Changes in charge distribution in ESI mass spectra for the reduced and oxidized forms indicate a change in the number of accessible protonation sites in the oxidized protein, and thus an altered structural conformation upon oxidation. Proteolytic digestion and MS analysis of the oxidized protein were reported to identify sites of disulfide cross-linking upon oxidation (Figure 1-8). Few experimental procedures were presented. Because most tryptic digest procedures require the reduction of disulfide bonds, the ability to determine the sites of oxidation is challenging and is not explained in this article. However, it is clear that ESI-MS can be a useful tool in examining structural changes in proteins based on change in their overall charge state.

Examination of proteins during most of the last decade focused on subjects that are soluble due to their ease of analysis. In 1998 Ball *et al.* presented a method of using

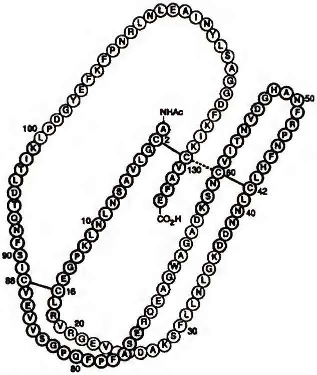


Figure 1-8. Schematic representation of the primary sequence of oxidized galactoside-binding lectin of bovine heart muscle. The predominant disulfide bonds identified from tryptic digestion followed liquid secondary ion mass spectrometry (LSIMS) are shown as solid lines between oxidized cysteine residues. A minor alternative disulfide bond is shown as a broken line. Figure was reproduced from Tracey *et al.* (68).

HPLC and ESI-MS to probe integral membrane-bound insoluble proteins (69) by probing bacteriorhodopsin (bR), its cysteine-containing mutants, and the G-protein coupled receptor, rhodopsin (Rh). The difficulty associated with analysis of integral membrane-bound proteins is that they and their cleavage fragments are amphiphilic molecules, which tend to aggregate, often irreversibly, and bind irreversibly to HPLC column packings. This method included the use of the organic soluble reagents tributylphosphine and vinylpyridine to reduce and alkylate the proteins prior to their removal from the membrane. Cleavage by cyanogen bromide was followed with subsequent analysis by HPLC/ESI-MS and MS^n. Using this method first on the previously mapped protein bR, then on its cysteine mutants, it was then applied to the more complex system of Rh. Rh had previously been partially mapped by MS, and sections of the sequence were unknown. Sequence elucidation had been considered the major obstacle to further study of Rh and its interactions with other signal transduction proteins with chemical modification and cross-linking experiments, and in this study Ball *et al.* reported the complete sequencing of Rh.

As the complexities of biochemical problems have grown so have the applications for which ESI-MS can be used. In 1999 Raida *et al.* (70) reported the mapping of peptides from human plasma filtrate using HPLC/ESI-MS. Characterization of complicated mixtures of proteins and peptides has frequently been accomplished with the well-established method of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (71-73). HPLC/ESI-MS can be used instead of the 2D-PAGE method due to its accurate and sensitive detection of small peptides. Human blood ultrafiltrate (HF) is a complex mixture of peptides in a wide range of concentrations and molecular weights. This broad range of peptides was mapped using HPLC/ESI-MS establishing a map of the peptides present in human blood ultrafiltrate. The use of HPLC/ESI-MS extended the range of analysis to lower molecular weights than the traditional 2D-PAGE for analysis of this complex mixture of peptides.

ESI-MS for Analysis of Chemical Modification and Bioconjugation in Proteins

The characterization of post-translational modifications in proteins has become a useful function of ESI-MS as demonstrated by the examples above. This analytical technique has then lent itself in recent years to the characterization of chemically modified proteins where the basis of chemical modification was described previously. The ability to examine chemical modifications to proteins and their implications on primary structure, residue activity, protein folding, interactions with other subunits, catalytic activity, and so on, is forwarded by such a rapid and highly sensitive method as

ESI-MS. Many researchers have turned to HPLC, ESI-MS, and MS^n as the primary analytical tools for the characterization of their chemically modified proteins.

One example of the use of this technology was reported by Akashi *et al.* (74). Their study of the guanosine triphosphate (GTP) binding protein, Ras and its protein substrate, Raf-1 Ras binding domain (Raf-1RBD), was carried out by chemical modification with glycinamide at acidic residues (Figure 1-9). Subsequent studies of interactions between the glycinamide-modified Ras protein and the substrate Raf-1 RBD resulted in information regarding the active and inactive forms of the Ras protein known to be related to a conformational change from the GDP- to the GTP-bound state. Chemical modification and HPLC/MS were used to extend the information already obtained by NMR and X-ray crystallography. The molecular weights of the modified Ras and the Raf-1 RBD were obtained by MS indicating limited modification to the proteins. To determine sites of modification, tryptic digests were performed and both MALDI and HPLC/ESI-MS were used to map the protein (Figure 1-10). The conclusions drawn indicated significant modification on one region of a peptide segment that was implicated as important for complex formation with the substrate protein. Certain problems with the study, however, were not addressed, including comparison of the modified protein peptide maps to a native peptide map, and there was no discussion on the relation of peptide concentration to MS ion peak intensity. Such issues have become apparent in

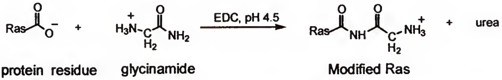


Figure 1-9. Schematic representation of the modification of guanosine triphosphate binding protein, Ras, with glycinamide at glu or asp residues. Both active (GTP-bound) and inactive (GDP-bound) forms of the Ras protein were modified.

research discussed below and indicate the care that must be taken when interpreting chemical modification data from peptide mapping using MS.

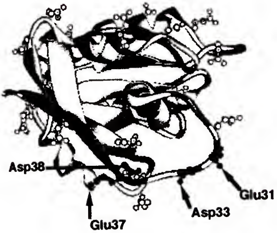


Figure 1-10. The tertiary structure of Ras protein with substrate bound obtained by X-ray crystallography. Balls and sticks indicate side chains of acidic amino acids. Those side chains indicated with a label are suggested to be the complex interface with Raf-1 RBD. Figure reproduced from Akashi *et al.* (74).

Another example of chemical modification used in conjunction with ESI-MS was reported by Zappacosta *et al.* (75). The subject of their study was the Minibody, a *de novo*-designed 61-residue metal-binding protein. The design of this protein contains a ß-sheet scaffold based on a heavy-chain variable domain structure of a mouse immunoglobulin. The general fold of the protein was already known, but detailed three-dimensional structure had been problematic because of the protein's low solubility. Proteolysis was used to examine exposed sites and flexible loops within the linking regions of the peptide chain between domains (Figure 1-11). Chemical modification of various residues with different modification reagents was performed followed by characterization of their effects on proteolysis using HPLC/ESI-MS. Lysine residues were modified with acetic anhydride, tyrosine residues with N-acetyl-imidazole, which targets the phenolic hydroxyl group, with tetranitromethane, which targets the aromatic ring, and arginine with 1,2-cyclohexanedione. Tryptic digests of the modified proteins and subsequent mapping by HPLC/ESI-MS provided selectivity information for lysine residues. Of the five lysines to be modified, two were found to be highly reactive, two were minimally reactive and only when the first two were modified, and one was not reactive. Such a system provided information on lysine accessibility and therefore folding of the protein scaffold. Modification of tyrosine and arginine residues yielded site of modification information along with qualitative selectivity information, while no mention was made of peak intensity correlation to concentration. This study is not only a good example of the current use of HPLC/ESI-MS as a tool for peptide mapping but also demonstrates some of the drawbacks in terms of the quantitative data that can be obtained.

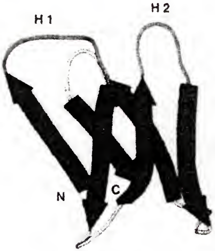


Figure 1-11. Secondary structure assignment of the Minibody (H1 and H2 designate hypervariable loops). Chemical modification at surface accessible basic residues allowed for the correlation of the relative reactivities with the position within the tertiary structure of the protein. Figure has been reproduced from Zappacosta *et al.* (75).

An example of chemical modification and ESI-MS used for probing active arginine residues in creatine kinase (CK) was reported by Wood *et al.* (76). Such an application is well suited for ESI-MS as qualitative data is sufficient for identifying residues of interest. CK is involved in energy metabolism in cells by catalyzing the reversible transfer of a phosphoryl group from phosphocreatine to MgADP to form creatine and MgATP. Modification of CK by phenylglyoxal (Figure 1-12) was followed by MS^n analysis or Lys-C digestion and ESI-MS analysis. The results of these studies implicated three arginine residues in the inactivation by phenylglyoxal and thus as pivotal in enzyme activity or folding.

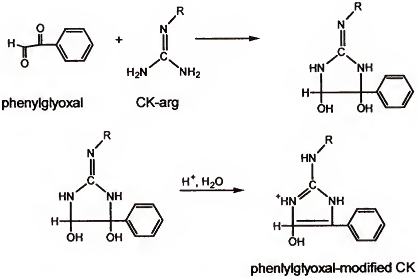


Figure 1-12. Reaction scheme of creatine kinase (CK) arginine residues with phenylglyoxal. Modification of three arginine residues by phenylglyoxal resulted in the inactivation of the phosphoryl group transfer activity of CK. Reaction scheme reproduced from Wood *et al.* (76).

Examples such as those mentioned above demonstrate the utility of ESI-MS for characterizing and mapping of chemically modified proteins. The quantification of selectivity of residues presents an obstacle that is explored further below. In spite of this limitation, use of ESI-MS can logically be extended to include probing of chemical modifications that can ultimately be linked to further functionalities. Such a joining of two functions by a chemical or biological means is termed "bioconjugation" (77).

Bioconjugation has been used for many purposes but most include alteration or enhancement of enzyme function. Perhaps the original example of bioconjugation of an enzyme to a functional group was reported by Levine *et al.* in 1977 (78). In this study the enzyme papain was reacted with brominated flavins to produce a covalent linkage at Cys-25 in the active site of papain (Figure 1-13). The result was a new semi-synthetic oxoreductase. The single free sulfhydryl in papain made the enzyme highly suitable for such a bioconjugation, as the site of modification was unequivocal. A complete review of these flavopapain derivatives was reported by Kaiser *et al.* in 1984 (79).

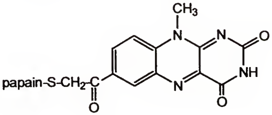


Figure 1-13. Structure of one flavin derivative covalently linked to papain at cys-25 resulting in a semi-synthetic oxoreductase, 7α-papain acetyl-10-methylisoalloxazine. Figure reproduced from Levine *et al.* (78).

In order to explore biological electron transfer in cytochrome *c*, Pan *et al.* (80) performed a bioconjugation between cytochrome *c* lysine residues and $Ru^{II}(bpy)_2CO_3$ to form a singly labeled $Ru^{II}(bpy)_2$(dcbpy-cytochome *c*) semi-synthetic system. The two-step bioconjugation involved the initial modification of lysine residues by mono-N-hydroxysuccinimide ester of 4, 4'-dicarboxy 2,2'-bipyridine (dcbpy). The derivatives were then separated by HPLC to obtain purified singly labeled protein. A further reaction was then accomplished by treatment with $Ru^{II}(bpy)_2CO_3$ (Figure 1-14). The

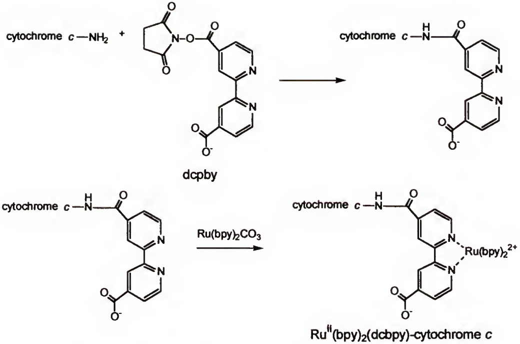


Figure 1-14. Schematic representation of the preparation of Ru^{II}(bpy)$_2$(dcbpy)-cytochrome *c* derivative. Bioconjugation was accomplished through modification of cytochrome *c* lysine residues with dcbpy. The subsequent addition of Ru(bpy)$_2$CO$_3$ allowed for the complexation of Ru(bpy)$_2^{2+}$ to the bipyridal ligand. Reaction schemes reproduced from Pan *et al.* (80).

luminescence emission was monitored for each enzyme derivatized at a different site. While this preparation produced singly modified semi-synthetic derivatives, the site of modification was determined by proteolytic digestion and amino acid sequencing. It is possible that ESI-MS would provide a more rapid analysis with higher specificity.

Another example of bioconjugation through chemical modification was reported by Albert *et al.* in 1992 (81). The generation of fluorohydrolase activity in an enzyme was accomplished by the bioconjugation of dimethyl pimelimidate through the chemical modifier hexamethylphosphoroamide (HMPA) to the enzyme, bovine pancreatic ribonuclease (RNase). The derivatized enzyme displays high fluorohydrolase activity, but the mechanism of derivatization and the site of modification are not known. ESI-MS would be a complement to such a study for the elucidation of further information about this semi-synthetic system.

Another type of bioconjugation recently explored is the introduction of a catalytic cofactor into the active site of an enzyme to prepare a new semi-synthetic catalyst. A review of bioconjugation with catalytic cofactors was presented by Distefano *et al.* (82) in 1998. In general, successful bioconjugations have been performed by either chemical modification of proteins that contain a unique residue susceptible to modification or by the introduction of such a residue by site-directed mutagenesis. Many examples of semi-synthetic enzymes prepared using these methods exist, including work by Ebright *et al.* (83), Sigman *et al.* (84-88), Schultz (89,90), Dervan (91,92) and Distefano (93).

The field of semi synthetic enzymes, while promising in terms of its potential, has been slow moving in terms of its production of a semi-synthetic system that has direct therapeutic application. Also, the use of the protein with the unique modifiable residue ultimately limits the number of base protein systems that can be exploited, and thus site-directed mutagenesis is required to introduce the unique modifiable residue. Based on these modification issues, it would be advantageous to use chemical modification of a protein that did not possess a uniquely modifiable residue followed by its separation and subsequent bioconjugation to another functionality of interest to broaden the number of potential protein candidates for derivatization. The ability to guide and track the bioconjugation of the protein is required and is possible with the use of HPLC and mass spectrometry.

The research project presented herein was performed for the bioanalytical method development of protein bioconjugation through chemical modification with a protein that does not possess a uniquely modifiable residue. HPLC and ESI-MS have been employed to guide and track the development. Results described provide a method that could potentially be applied to any carefully chosen protein and modification reagent for the development of a semi-synthetic enzyme.

CHAPTER 2
CHEMICAL MODIFICATION OF RIBONUCLEASE A

Introduction

Development of a functional bioconjugate requires initial selection of a protein to be modified. As the development of a bioconjugation system was pursued in this research, the choice of base protein was limited only to an enzyme that was well-characterized, readily available, and susceptible to chemical modification. These requirements were satisfied with bovine pancreatic Ribonuclease A (RNase A).

RNase A hydrolytically cleaves the phosphate backbone of RNA and has been the object of groundbreaking work in the basic understanding of proteins and enzymes. It has been the subject of research in protein folding, stability, enzymatic activity, structure and evolutionary development. Numerous reviews on this protein include those by Anfinsen *et al.* (94), Raines (95), and D'Alessio and Riorian (96). Original work on bovine pancreatic Ribonuclease A was begun by Jones in 1920 (97), and crystallization of the protein was first accomplished in 1939 by Kunitz (98). Since then, many significant papers have been published regarding the activity (99), chromatographic separation and purification (100,101) assay development (102,103), and investigation of active site residues (104,105). Crystallographic studies include work by Kartha *et al.* (106), Carlisle *et al.* (107) Wlodawer *et al.* (108), and Kim *et al.* (109). Work has also been reported on kinetic and thermodynamic studies of RNase A (110), anion binding and pH electrostatic effects (111), and investigations by NMR (112-114).

Significant numbers of reports have focused on the chemical modifications of RNase A that include early affinity labeling with pyridoxal phosphate (115), carboxymethylation of active site histidine residues (116), the targeting of arginine residues by the modification reagent 1,2-cyclohexanedione to study proteolytic digestion (117), modification of various residues for the study of interaction between RNase A and its human placental inhibitor (118,119), the study of glycation of amino groups as a model for glycation in proteins (120), and the probing of RNase A phosphate binding subsites (121,122).

Along with crystallography, NMR and sequencing techniques, RNase A has been well-characterized by mass spectrometry. Mass spectrometric studies have included investigations on the protein conformation and the differences between RNase A and Ribonuclease B (RNase B), which is a glycosylated form of RNase A (123,124). Non-covalent interactions of substrates, inhibitors, and adducts with RNase A and variants of RNase A have also been studied by ESI (125-127). ESI-MS was used to study non-covalent interactions, thermal denaturation and entropy changes of RNase S (128-130).

Mass spectrometry has also been used to characterize chemical modifications in RNase A. Glocker *et al.* (131) reported the acetylation and succinylation of RNase A using HPLC and mass spectrometry to identify sites of modification and to quantify the reactivity of those sites.

The accessibility of large quantities of RNase A along with the widely accepted knowledge of its structure and activity made RNase A well suited for bioconjugation method development. The susceptibility of RNase A to chemical modification has been well-documented as described above. The protein contains 10 lysine residues that can be chemically modified by exploiting the nucleophilicity of their ε-amino groups and the

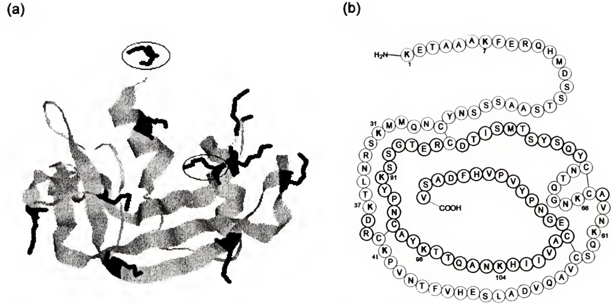


Figure 2-1. Ribonuclease A (RNase A) (a) Three-dimensional structure represented by a 'ribbon' diagram. The ten Lysine residues are represented as sticks. Lys-1 and Lys-41 are circled. (b) Primary sequence of RNase A. Lys-1 has two possible sites of modification, the ε- and α-amino groups. All lysine residues may be modified under certain reaction conditions.

terminal α-amino group (see Figure 2-1). The pKa of the α-amino terminus group has been reported as 6.6, the ε-amino group for active site lysine 41 has been reported as 8.6 and the ε-amino groups on the rest of the protein range from 10.6 to 11.2 (113). The variability in the lysine pKa's presents the possibility that certain amino groups may be selectively modified.

The reagent 2-iminothiolane (Traut's reagent) was the first reagent chosen to modify RNase A. The reaction scheme is shown in Figure 2-2. Traut's reagent has been used for the study of protein structures by reaction with lysine residues, followed by oxidation to form a disulfide bond between the imidate groups (132). The bifunctionality of the imidate label was to be used later in the bioconjugation of RNase A as a linker to

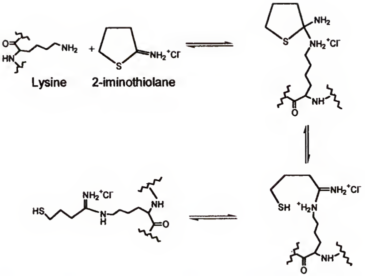
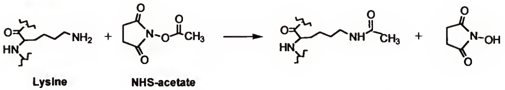


Figure 2-2. Reaction scheme of Traut's reagent (2-iminothiolane) with an ε-amino group of lysine. Nucleophilic attack by the ε-amino group of lysine results in the addition of an imidate linkage to a terminal thiol. The imine group in the functionality maintains a positive charge, thus conserving the charge at that site on the protein after modification.

another group of designed functionality. Ideally, the sulfhydryl would be reacted with a thiol that had been derivatized to contain a metal binding group such as bipyridyl amine. Modification of RNase A with this two-step process would result in covalent attachment of a metal binding cofactor to RNase A. Separation of RNase A modified with Traut's reagent was found to be problematic because of the positive charge that was maintained on the functionalized residue after modification.

As a control study, a new modification reagent was chosen that would neutralize the positive charge in lysine residues upon reaction. The reagent N-hydroxy succinimide acetate (NHS-acetate), shown in Figure 2-3, was synthesized according to the method of Lindsay and Shall (133) and, subsequently used to modify RNase A. Mass spectrometric examination was employed to verify the modification of the protein. A procedure for separation of RNase A modified with different numbers of acetate groups was also developed using cationic exchange. Preliminary results of this control modification and separation indicated the possibility of separation of singly modified RNase A (see below). With these preliminary results, a new modification reagent was chosen that would possess further functionality.

Scheme 1



(1)

Figure 2-3. The N-hydroxy-succinimide group of NHS-acetate reacts with nucleophilic amino groups (Scheme 1).

A modification reagent possessing bifunctionality was desired for bioconjugation once mapping of the modified RNase A system had been established. Two heterobifunctional cross-linking reagents were chosen; first, N-succinimidyl-3-[2-pyridyldithio] propionate (SPDP), and second, 4-succinimidyloxycarbonyl-methyl-ε-[2-pyrdyldithio]-toluene (SMPT), both shown in Figure 2-4. SPDP and SMPT have been extensively used in the development of immunochemical therapeutic reagents. The mass change upon modification of RNase A with SPDP (197.28 Da) is equal to that of two phosphate adducts, making the unequivocal determination of modification difficult. For this reason, the reagent SMPT was used. SMPT's chemical functionality is the same as SPDP's, but the mass change (273.34 Da) upon modification of RNase A allowed for unequivocal determination of the numbers of SMPT groups added.

SMPT has been utilized for the preparation of immunotoxins, linking a cell-binding component to a potent toxin to target Hodgkin's lymphoma (134-136). SMPT has also been used in a similar manner for the development of thrombolytic and

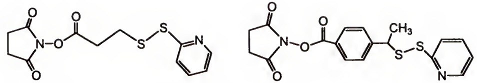


Figure 2-4. Heterobifunctional cross-linking reagents. a) N-succinimidyl-3-[2-pyridyldithio]propionate (SPDP). b) 4-succinimidyloxycarbonyl-methyl-ε-[2-pyrdyldithio]-toluene (SMPT).

antiplatelet reagents (137). The N-hydroxy-succinimide group of SMPT reacts with nucleophilic amino groups on proteins (Figure 2-5, Scheme 1). The covalently linked SMPT provides a dithio group that can be exchanged with a thiol compound to further derivatize the protein (Figure 2-5, Scheme 2).

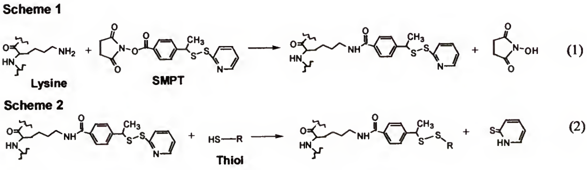


Figure 2-5. The N-hydroxy-succinimide group of SMPT reacts with nucleophilic amino groups (Scheme 1). The covalently linked SMPT provides a dithio group that can be exchanged with a thiol compound to further derivatize the protein (Scheme 2).

Optimization of cation exchange separation procedures enabled investigation of the numbers of SMPT reagents added to the RNase A. Subsequent analyses were performed on HPLC/ESI/QIT-MS, where QIT refers to quadrupole ion trap. HPLC online with ESI/QIT-MS was used to investigate the separation of modified RNase A based on numbers of modifications. The multi-dimensional separations developed for this chemically modified protein followed by mass spectrometric analysis demonstrate their applicability for isolation and characterization of other potential bioconjugate systems.

Experimental Procedures

General Reagents

All reagents were reagent grade unless otherwise specified. All buffers were purchased from Sigma (St. Louis, MO). Water was purified by a Sybron/Bamsted Module Deionization System.

Ribonuclease A Purification

Ribonuclease A, type 1-AS from bovine pancreas, was purchased from Sigma. Purification was accomplished by two methods.

Purification method 1

RNase A was purified with an Amberlite cationic exchange column (200-400 mesh). The column was eluted with a linear gradient from 0.14 M to 2.0 M sodium phosphate, pH 6.95. The last eluted peak was collected and rechromatographed on a Sephadex G100 in 0.2 mM tris (hydroxymethyl)-aminomethane buffer (tris), pH 8.0, to remove salts. The protein was dialyzed into 0.1% trifluoroacetic acid (TFA) and lyophilized for long-term storage. The concentration of RNase A in solution was determined by UV/visible spectroscopy (Perkin-Elmer 390), $\epsilon = 9800\ \text{cm}^{-1}\ \text{M}^{-1}$.

Purification method 2

RNase A was purified using a Bio-Rex 70 cationic exchange resin from Bio-Rad (Hercules, CA). The column (0.7 x 70 cm) was eluted with an exponential gradient from 10 mM to 160 mM ammonium phosphate, pH 7.2 at a flow rate of 0.6 mL / min. The eluate was monitored at 229 nm. The last eluted peak (RNase A) fractions were collected, concentrated by Amicon filtration (MW=3500) and desalted by using a Sephadex G50 (Sigma) column with 0.2 mM tris buffer (Acros, New Jersey), pH 8.0.

The protein was dialyzed into H_2O and lyophilized for long-term storage. The concentration of RNase A in solution was determined by UV/visible spectroscopy (Perkin-Elmer 390).

Kinetic Assay of RNase A Activity

Enzyme activity was determined by the methods of Crook *et al.* (102) and Eftink *et al.* (138). Briefly, the RNase A catalyzed hydrolysis of cytidine 2', 3'-cylic monophosphate (2,3-cCMP) at 25°C was monitored by following the increasing concentration of the reaction product, 3-CMP, at 295 nm. The pseudo first-order rate constant, k_{obs}, was determined. Under the assay conditions, native RNase A had a rate constant (k_{obs} / [E]) of $1.2 \pm 0.5 \times 10^3\ M^{-1}\ s^{-1}$. Eftink *et al.* (138) have reported a rate constant of $2.57 \times 10^3\ M^{-1}\ s^{-1}$.

Modification of RNase A with Traut's Reagent

Traut's Reagent, 2-iminothiolane, was purchased from Pierce. A stock solution of Traut's Reagent was prepared by dissolving 3.44 mg of the reagent in 0.05 mL of 1 M 2-[N-morpholino]ethanesulfonic acid (MES) buffer, pH 6.5. The RNase A was dissolved in 2 mL of 50 mM MES buffer, pH 6.5. The 0.05 mL of Traut's Reagent was added to the RNase A solution and left to incubate for 8+ hours at 4°C. The reaction mixture was then dialyzed against 50 mM MES buffer, pH 6.5, followed by dialysis against 49% H_2O / 49% methanol / 2% acetic acid for electrospray mass spectrometry. These conditions were optimized with regards to pH, buffer salt, buffer concentration, temperature and molar ratios of modifying reagent and protein.

NHS-Acetate Synthesis

2.86 mL acetic acid (17.4 M) was dissolved in 30 mL dichloromethane (Fisher). In a separate solution, N-hydroxy succinimide (5.76 g) was dissolved in 10 mL dioxane with heating and then added to the dichloromethane solution with stirring. 10.32 g of dichlorohexylcarbodiimide (DCC) was also added. The pale yellow solution was stirred overnight at 4°C followed by 2 hours at room temperature. The solution was then filtered with a Buchner funnel and the filtrate was quickly concentrated by vacuum roto-evaporation. The solid was recrystallized in ethyl acetate and yield was 50%.

Modification of RNase A with NHS-Acetate

RNase A and NHS-acetate were incubated in an 80% tris buffer (10 mM, pH 8.0) / 20% DMF solution for 2 hours at room temperature. A 1:1 molar ratio of RNase A to NHS-acetate was used. The NHS-acetate stock was prepared in DMF and then diluted with tris buffer. The final reaction mixture was 1.5 mL. After incubation, the reaction mixture was either dialyzed against 49% H_2O / 49% methanol / 2% acetic acid for electrospray mass spectrometry or applied to a Bio-Rex 70 column. Elution gradient for the Bio-Rex 70 was the same as that used for native RNase A in purification method 2. Fractions were collected, concentrated and dialyzed into 49% H_2O / 49% methanol / 2% acetic acid for electrospray mass spectrometry.

Modification of RNase A with SPDP

The modifying reagent, N-succinimidyl-3-[2-pyridyldithio]propionate (SPDP), was purchased from Pierce. The molar ratios of SPDP to RNase A were 1:1, 2:1 and 4:1. The SPDP stock was prepared in DMF and then diluted with tris buffer (10 mM, pH 8.0). The RNase A and SPDP were incubated in 80% tris buffer (10 mM, pH 8.0) / 20% DMF for 3½ hours at room temperature. The final reaction mixture had a volume of 1.5 mL. After incubation, the reaction mixture was applied to a Bio-Rex 70 column. The elution gradient was the same as that used for native RNase A. Fractions were collected, concentrated and dialyzed into 49% H_2O / 49% methanol / 2% acetic acid for electrospray mass spectrometry.

Modification of RNase A by SMPT

Purified RNase A was dissolved in 50 mM N- [2hydroxyethyl] piperazine-N'-[3-propane-sulfonic acid] (EPPS) buffer (Sigma) at pH 7.0 or 8.0. RNase A was modified at nucleophilic lysine residues with SMPT (Pierce). The reaction was run in 20% acetonitrile (Fisher) / 80% 50 mM EPPS buffer at pH 7.0 or 8.0 with a final reaction volume of 1.5 mL. A molar ratio of 2:1 was used at pH 8.0 (4:1 at pH 7.0) for SMPT to RNase A, with an incubation time of 6.5 hours at room temperature. Fractions were collected, concentrated and dialyzed into 49% H_2O / 49% methanol / 2% acetic acid for electrospray mass spectrometry.

Separation of Modified Ribonuclease A Products

The reaction solution was applied directly to a Bio-Rex 70 column (0.7 x 70 cm), and was eluted overnight with an exponential gradient of 10 mM to 160 mM ammonium phosphate at a flow rate of 0.3 mL/min. The elution was monitored at 229 nm with an Isco absorbance monitor, UA-5, and the fractions were collected, concentrated and either lyophilized and reconstituted in 49% methanol / 49% H_2O / 2% acetic acid for mass spectrometric analysis. If the fractions were to be further separated by HPLC, they were collected and concentrated to 250 μL by Amicon filtration, MW cutoff = 3500.

HPLC Separation of Modified RNase A Offline

Some Bio-Rex 70 separated SMPT modified RNase A fractions were further separated on a Phenomenex Jupiter 5μ C18 column (Torrance, CA) (4.6 mm i.d. x 250 mm; guard column, 4.6 mm i.d. x 30 mm, 300 Å pore). Buffer A consisted of 1% stock buffer (below), 10% acetonitrile (HPLC grade, Fisher), 89% H_2O and buffer B was 1% stock buffer, 75% acetonitrile, and 24% H_2O. The stock buffer consisted of 7.5% trifluoroacetic acid (Fisher), 50% triethanolamine (Fisher), 25% acetonitrile and 62.5% H_2O. The column was equilibrated with 5% buffer B at 1 mL/min. The elution was as follows: 0 min (A:B=95:5), 5 min (A:B=95:5), 20 min (A:B=70:30), 30 min (A:B=65:35), 40 min(A:B=25:75); 50 min (A:B=95:5). The various forms of modified RNase A were detected by their absorbance at 229 nm. The peak fractions were collected, concentrated, lyophilized and reconstituted in 49% methanol / 49% H_2O / 2% acetic acid for mass spectrometric analysis.

Electrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

ESI/FTICR-MS data were collected by G. H. Lisa Lang and Linda S. Nichols. All ESI/FTICR mass spectrometric experiments were performed with a Bruker BioAPEX external source shielded 4.7-Tesla FT-ICR mass spectrometer (Bruker Analytical Systems, Inc., Billerica, MA) equipped with a Bruker Xmass 4.0.3 data system. A detailed description of the instrument can be found elsewhere. (139,140) The electrospray source was a modified Analytica electrospray source (Analytica of Branford, Branford, CT). The stainless steel desolvation capillary was held at ground potential and heated to 120°C. The FTICR cell pressure was 1×10^{-9} torr. The samples were sprayed at a rate of 1 μl/min. The electrospray needle (2950 V) was located at a distance ca. 1 cm from the capillary. The capillary and skimmer voltage were adjusted to values which maximized the signal intensities for the ions of interest, and typical values were 100 and 10 volts for the capillary and skimmer, respectively.

HPLC/Quadrupole Ion Trap Mass Spectrometry

High performance liquid chromatography / electrospray ionization / quadrupole ion trap / mass spectrometry (HPLC/ESI/QIT-MS) data were collected by Jodie V. Johnson. HPLC/ESI/QIT-MS data were acquired on a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) operated in the electrospray ionization (ESI) mode. Typical operating parameters included nitrogen sheath and auxiliary gases of 70 and 10 (unitless), respectively, a heated capillary temperature of

235°C, and an ion spray voltage of 3.5 kV. MS data were acquired with the normal m/z 50-2000 range as well as with the extended m/z range (up to m/z 4000) offered with the LCQ's advanced scan function software.

The mobile phase was introduced by a Beckman Instruments (Fullerton, CA) System Gold model 126 pump. The binary HPLC mobile phase system used is the same as described previously for offline HPLC. Chromatographic separation was achieved with either a Phenomenex Jupiter 5µ C18 column size (column and gradient described above) or a Waters (Milford, MA) Symmetry C18 (2.1 mm i.d. x 150 mm, 5 µm particles, 100 Å pore). A typical gradient at 0.2 mL/min for the Waters column was: 0 min (A:B=95:5), 1 min (95:5) 41 min (5:95), and 45 min (5:95). The column effluent was split prior to the ESI source with approximately 70-80% of the effluent going to waste.

Results and Discussion

Purification of RNase A

Bovine pancreatic RNase A is a well-characterized protein (MW 13681 Da) that hydrolytically cleaves RNA. In order to pursue extensive modification and mapping studies for characterization of the protein derivatives and evaluation of their potential as bioconjugates, a large amount of RNase A starting material was needed. It was expected that modification, separation, and analysis would require not only significant amounts of RNase A, such as gram quantities, but also highly purified protein. For this reason lower grade bovine pancreatic RNase A was purchased from Sigma and purified according to the method by Crestfield, published in 1963 (101).

In eukaryotic cells, in addition to RNase A, ribonuclease is also expressed as RNase B, which has an oligosaccharide chain covalently linked to Asn 34 (110). Mannose rings enable RNase A to be separated from RNase B by virtue of their differing dipole moments and ability to interact with ionic exchange resin. Consequently, cation exchange separation with Amberlite resin and linearly increasing salt gradient separates RNase B (eluting early) and RNase A (eluting later) (Figure 2-6). RNase A was collected and rechromatographed using a size-exclusion column (G-50) to desalt. Kinetic activity was verified as described.

A problem with this purification method arose when, as a control, RNase A that had been purified by method 1 was applied to the Bio-Rex 70 cationic exchange column that was used for separation of modified protein. It was found that under the conditions used for the Bio-Rex 70 elution, other unidentifiable residual peaks appeared (retention times between 12 and 15 ½ hours) prior to the expected retention time of RNase A (retention time between 16 and 18 hours) (Figure 2-7). The large peak at the retention time of RNase A was collected and rechromatographed under the same conditions to verify removal of all residual peaks (Figure 2-8).

The above results indicated that Bio-Rex 70 chromatography must be used as part of the purification procedure. It was then attempted to purify the RNase A by using the Bio-Rex 70 column and omitting the Amberlite resin originally used by Crestfield (101). Such a separation of impure RNase A yields numerous peaks indicating RNase A of varying degrees of purity in the originally purchased sample (Figure 2-9). Collection of the peak with the longest retention time and re-separation on the Bio-Rex 70 indicated elimination of unidentified residual peaks. Therefore, the step of Amberlite chromatography was eliminated.

Along with kinetic assays of activity, purity of RNase A was examined by ESI/FTICR-MS. The multiply charged protein with the highest peak intensity is $[M+8H]^{8+}$ charge state, with 8 protons attached to the protein. Mass spectra units are in m/z, and

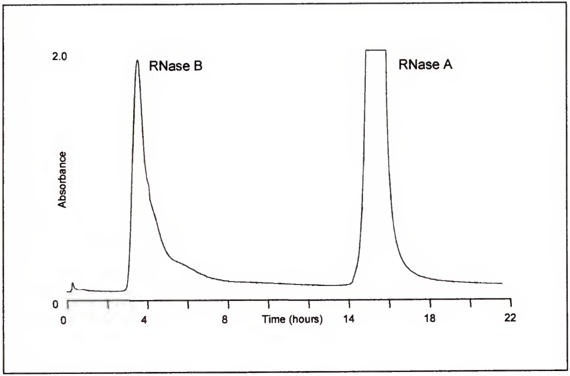


Figure 2-6. Purification of RNase A by Amberlite cationic exchange. The protein was eluted with linear salt gradient (0.14 M to 2.0 M) of sodium phosphate, pH 6.95. Separated is RNase B (eluting early) from RNase A (eluting later). The second peak (RNase A) was rechromatographed on Sephadex G100 resin to desalt.

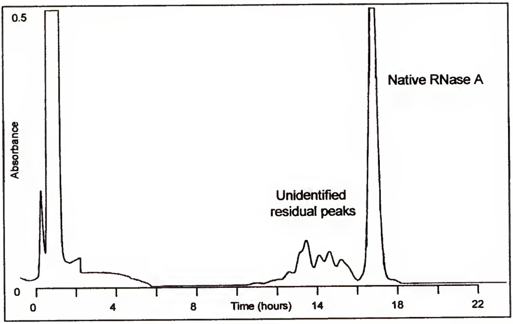


Figure 2-7. For purposes of control, RNase that had been purified according to method 1 was incubated in the modification reaction mixture (80% tris / 20% DMF) in the absence of modification reagent followed by chromatographic separation on Bio-Rex 70 resin. Unidentified residual peaks appeared prior to the expected retention time of the native RNase A.

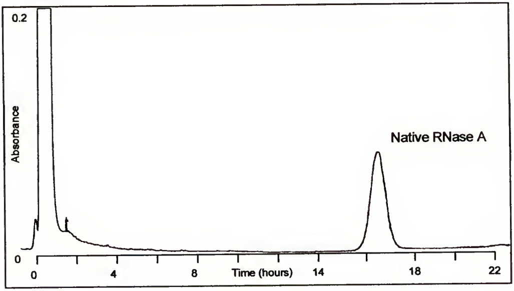


Figure 2-8. The large peak from Bio-Rex 70 separation of purified RNase A (Figure 2-7) was collected, incubated again in the modification reaction mixture (80% tris / 20% DMF) in the absence of modification reagent, and rechromatographed on Bio-Rex 70 resin. All unidentified residual peaks were removed.

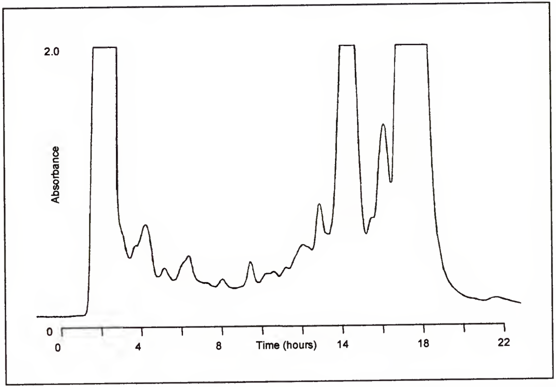


Figure 2-9. Purification of RNase A on Bio-Rex 70 cationic exchange resin. Removal of RNase B and other unidentified impurities was accomplished without the use of the Amberlite cationic exchange. The peak that eluted last (RNase A) was collected, concentrated and rechromatographed on Sephadex G50 to desalt.

multiplication by the charge state results in the exact mass of the protein (Figure 2-10). The first peak in the charge state envelope is native RNase A. Naturally occurring phosphate adducts were also seen as peaks at increasing m/z in the charge state envelope (123,125). High-resolution scans of a given peak revealed the isotopic pattern of that molecular ion (Figure 2-10 inset). The first peak in the isotopic envelope is the ^{12}C isotope followed by the addition of one ^{13}C for every following peak. The m/z difference between these peaks is 1/8 revealing its inverse as the charge state, $[M+8H]^{8+}$. This mass spectral data has been used for comparison of mass changes upon modifications with various reagents.

Modification of RNase A and Separation of RNase A Derivatives

RNase A contains 10 lysine residues, resulting in a total of 11 amino groups, including the α-amino terminus that can be modified by reaction with the imidate group of Traut's reagent (Figure 2-4) and the N-hydroxy-succinimide (NHS) groups of NHS-acetate, SPDP and SMPT (Figure 2-5). Lysine residues 1, 7 and 41 have a higher propensity for modification by reagents previously investigated as a result of surface accessibility and reduced pKa's (120-122,131). Based on the selectivities of lysine residues reported for other modification reagents, the reagents used here were evaluated in terms of their overall reactivity with lysine residues.

Modification with Traut's reagent

The first reagent used to modify RNase A was Traut's reagent. The reagent was chosen for its commercial availability, low cost, and the product that would result from reaction with RNase A lysine residues. As seen in Figure 2-1, the reaction product is an RNase A amino group derivatized to contain a terminal sulfhydryl. The sulfhydryl on the modification would be cross-linked to a functionalized ligand once the chemical derivatization of RNase A had been well characterized.

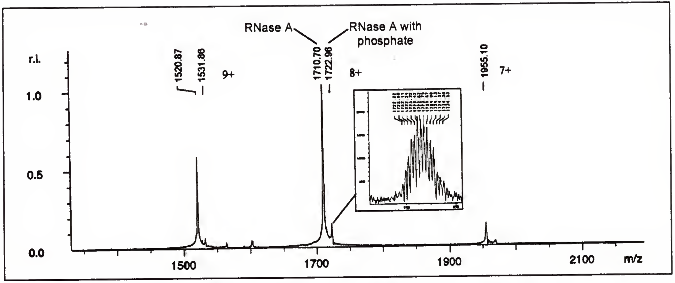


Figure 2-10. ESI/FTICR-MS of purified native RNase A. The most predominant charge state is $[M + 8H]^{8+}$, indicating the addition of 8 protons to the RNase A. M/z 1710.70-$[M + 8H]^{8+}$ corresponds to native RNase A ((1710.70)8 – 8(1.00794) = molecular mass of RNase A). One phosphate noncovalent adduct is represented by m/z 1722.96-$[M + 8H]^{8+}$. Inset) High-resolution scan of RNase A + 1 phosphate adduct, showing the isotopic pattern of single additions of ^{13}C to the monoisotopic m/z 1722.86-$[M + 8H]^{8+}$. The m/z difference between each isotopic peak is 1/8. The inverse is 8 and equals the charge state of the ion.

Chemical modification of RNase A by Traut's reagent was done according to the aforementioned experimental conditions. Reaction parameters were varied to maximize conversion of native RNase A to singly modified RNase A. Parameters varied included buffer (tris or MES), pH (6,7,8), and incubation time (20 minutes to 8+ hours). Variation in solution in which the RNase A and Traut's sample was applied to the ESI-MS was also pursued (methanol / H_2O / acetic acid vs. methanol / H_2O). The result of this array of controlled parameter variations was that RNase A appeared to be unmodified by Traut's reagent, or modified with many imidate reagent groups. An example of a typical ESI-MS of RNase A modified by Traut's reagent is seen in Figure 2-11. The most intense charge states typically seen in native ESI-MS spectra of RNase A, $[M + 7H]^{7+}$, $[M + 8H]^{8+}$, and $[M + 9H]^{9+}$, are maintained in the Traut's modification spectrum indicating general structural integrity of the protein. (Note that increase in ESI-MS charge states indicates protein unfolding due to increased numbers of protonation sites and decreased charge repulsion over the protein surface.) It is clear from Figure 2-11 that significant numbers of modifications have occurred when compared to the native RNase A in Figure 2-10. Resolution of the $[M + 8H]^{8+}$ state revealed covalent additions of at least six reagent groups and various numbers of phosphate adducts.

The high reactivity of RNase A for Traut's reagent would require the reaction products to be separated by liquid chromatography. Upon modification with Traut's reagent, the positive charges at lysine residues are retained due to the charge on the imidate group. Therefore, separation of RNase A molecules modified with different numbers of Traut's reagent groups would not be separable by normal-phase, reverse-phase, or ionic exchange chromatography. Because these separation methods were readily available, a different modification reagent was chosen that would neutralize the positive charge on the lysine upon modification and thus, allow for separation by ionic

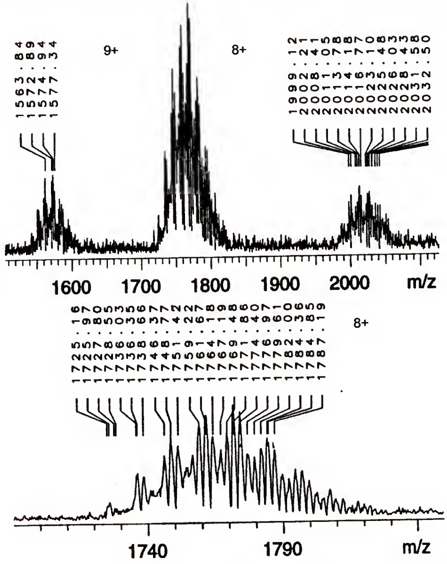


Figure 2-11. ESI/FTICR-MS of RNase A modified with Traut's reagent (2-iminothiolane). Predominant charge states are $[M + 7H]^{7+}$, $[M + 8H]^{8+}$ and $[M + 9H]^{9+}$. Numerous additions of imidate groups and phosphates are represented by the multiple peaks within each charge state. For example, m/z 1764.18-$[M + 8H]^{8+}$ corresponds to four imidate covalent additions to native RNase A. M/z 1776.97-$[M + 8H]^{8+}$ corresponds to five imidate covalent additions to native RNase A. The other peaks in the spectrum correspond to combinations of imidate additions and phosphate adducts.

exchange LC or HPLC. Rapid analysis by ESI-MS also demonstrated over-modification of RNase A by Traut's reagent. The choice of another modification reagent with a decreased electrophilicity may be less reactive with the lysine residues, resulting in selective modification at certain sites on the protein.

Modification with NHS-acetate

NHS-acetate was synthesized according to the method by Lindsay and Shall (133) and was used to modify RNase A through nucleophilic attack by lysine residues. The reaction product was an acetyl group covalently attached to the lysine residue that neutralized the positively charged amino group. Although no further functionality was introduced with the acetate, such neutralization presented the possibility of separation of reaction products by liquid chromatography. The acetate-modified RNase A was used to develop liquid chromatography separation conditions to be used later for separation of RNase A modified with reagents possessing greater functionality. Reaction conditions for the modification reaction were optimized through trial and error with the main obstacle being full dissolution of the reagent NHS-acetate in the reaction buffer. Dissolution was achieved when DMF was used to dissolve the NHS-acetate followed by dilution with tris buffer for a final solution ratio of 20% DMF/80% tris buffer. RNase A was also soluble in the reaction mixture as it was dissolved first in tris, and diluted with DMF.

Separation was accomplished using a procedure described by Durham *et al.* (141). The resin Bio-Rex 70, a weakly cationic exchange resin was used. The result of development of an elution gradient for separation of acetate-modified RNase A reaction products was a positive, exponentially increasing gradient of sodium phosphate. The column elution was further optimized to run overnight, allowing time for the structurally similar reaction products to separate. A chromatogram of a Bio-Rex 70 separation of the reaction mixture of RNase A and NHS-acetate is shown in Figure 2-12. Fractions for separated peaks were collected and examined by ESI-FTICR-MS. The mass spectrum for each resolved peak in Figure 2-12 is shown in Figures 2-13(a - e). The mass change for one modification is 42.03 Da. At the $[M + 8H]^{8+}$ state the m/z change is equal to 5.25. Clearly shown is the decreasing number of acetate modifications with increasing retention time. The neutralization of a lysine residue upon modification reduces its interaction with the anionic stationary phase resulting in reduced retention time. The native RNase A, peak E (Figure 2-12) is the most highly charged, being retained the longest.

Predominantly seen in the fractions from the Bio-Rex 70 elution were single and double modifications by NHS-acetate. Higher numbers of acetate additions may have caused any over modified protein to elute in the void volume of the column or possibly in what appears to be an unseparated region in the Bio-Rex chromatogram that eluted prior to any separated peaks. Also seen in figure 2-13 (e) is contamination considered to be polyethylene glycol polymers (PEGs). This type of contaminant appears in many mass spectra of biological molecules as the preparation of the samples usually requires using containers (for example, plastic test tubes, dialysis bags, etc.) that may contain these polymers. HPLC can be used to remove PEGs from samples of interest. HPLC was not used in the preparation of the acetate modified RNase A shown in Figure 2-13, and PEGs are present in the native peak E.

Because the goal of the separation was isolation of singly modified protein, the fractions that were found by ESI-FTICR-MS to contain predominantly single modifications demonstrated the feasibility of such an isolation. Since the acetate modification did not contain any functionality for further derivatization of RNase A,

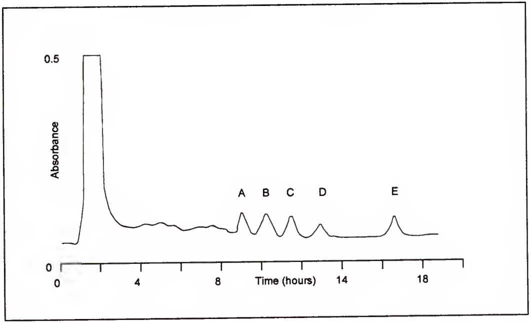


Figure 2-12. Bio-Rex 70 chromatogram of RNase A modified with NHS-acetate. Column was eluted with an increasing exponential gradient of ammonium phosphate, 10 mM to 160 mM, at pH 7.2 and 4°C. Acetate addition to lysine residues results in neutralization of positive charge. Decreasingly modified RNase A elutes with increasing retention time. Unmodified RNase A (peak E) elutes last as the most highly charged species.

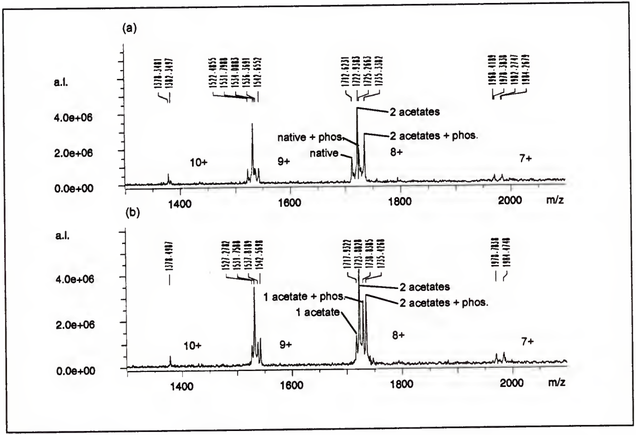


Figure 2-13. ESI/FTICR-MS of each peak from Bio-Rex 70 chromatogram in Figure 2-12. a) Mass spectrum of peak A. Most abundant peak, m/z 1722.9-$[M + 8H]^{8+}$ corresponds to addition of 2 acetate groups. Some native RNase A is present in the fraction as well as phosphate adducts. b) Mass spectrum of peak B. Addition of 2 acetate groups is the predominant peak with single addition of acetate also present, m/z 1717.9-$[M + 8H]^{8+}$ with and without phosphate adducts.

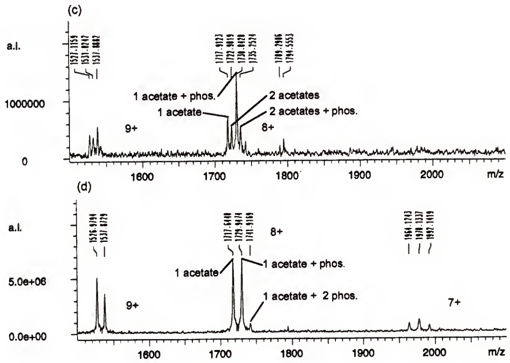


Figure 2-13 continued. c) Mass spectrum of peak C. Single acetate addition peak is the most abundant with double acetate addition peak present, both with and without phosphates. d) Mass spectrum of peak D. Only single addition of acetate present with and without phosphates.

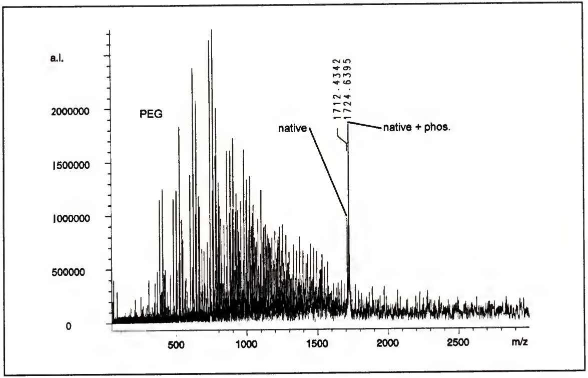


Figure 2-13 continued. (e): Mass spectrum of peak E, native RNase A present with and without phosphate. Also seen is contamination of low molecular weight polymers, typically polyethylene glycol. Contamination usually arises from trace detergents in glass and plastic ware in which sample is prepared.

modification was pursued with a new reagent that would allow for further functionality to be incorporated into the RNase A later.

Modification with SPDP

The reagent SPDP was the next modification reagent used with RNase A. Its commercial availability as well as its heterobifunctionality made it a good candidate for cross-linking the RNase A to a functionalized cofactor. The mechanism of reaction of SPDP with RNase A is the same as for NHS-acetate and RNase A. SPDP neutralized lysine residues upon reaction, as did NHS-acetate; therefore, the separation procedure used for the NHS-acetate could be optimized with the SPDP/RNase system. Optimization of the reaction conditions for conversion of RNase A into singly modified RNase A without over-modification was also pursued.

Optimization conditions were complicated by an unusually impure lot of RNase A. In the RNase A purification section previously described, unidentifiable residual peaks were discovered during control RNase A elutions on the Bio-Rex 70 column. This control study was performed only when the SPDP modification peaks appeared to have unexplained contamination. The unidentified residual peaks were co-eluting with the SPDP-modified RNase A fractions from the Bio-Rex 70 column altering the elution chromatogram. One example of a chromatogram where the residual peaks were present along with the SPDP-modified RNase peaks is shown in Figure 2-14. An example of the SPDP-modified RNase A elution chromatogram with the residual peaks removed is shown in Figure 2-15.

Another complication arose with the analysis of these SPDP-modified RNase A fractions by mass spectrometry. Upon modification with one SPDP group, the mass change to the RNase A was an increase of 197.28 Da. At the $[M + 8H]^{8+}$ state the m/z change is equal to 24.66. Typically seen in the mass spectrum of native RNase A are

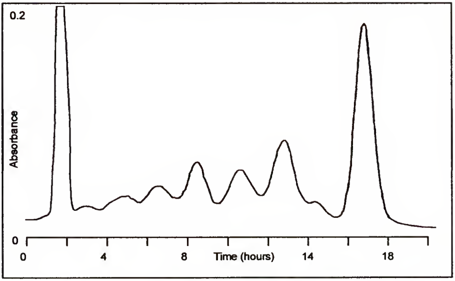


Figure 2-14. Bio-Rex 70 chromatogram of RNase A that had been purified by method 1 followed by modification with SPDP. Column was eluted with an increasing exponential gradient of ammonium phosphate, 10 mM to 160 mM, at pH 7.2 and 4°C. Separation of SPDP-modified RNase A fractions was poor due to contamination by unidentified residual peaks as in Figure 2-7.

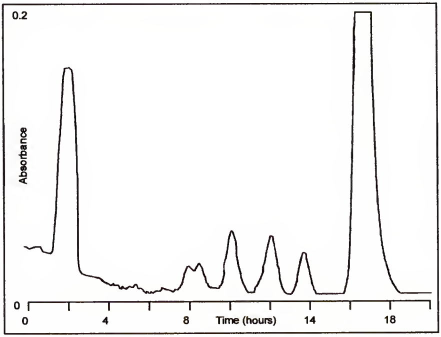


Figure 2-15. Bio-Rex 70 chromatogram of RNase A that had been purified by method 2 followed by modification with SPDP. Column was eluted with an increasing exponential gradient of ammonium phosphate, 10 mM to 160 mM, at pH 7.2 and 4°C. Resolution of SPDP-modified RNase A fractions was improved due to previous removal of unidentified residual peaks as in Figure 2-8. SPDP modification to lysine residues results in neutralization of positive charge. Decreasingly modified RNase A elutes with increasing retention time.

phosphate groups noncovalently bound to the protein phosphate binding sites (discussed previously). One phosphate group addition results in an increasing mass change of 97.99 Da (m/z change = 12.24 at the $[M + 8H]^{8+}$ state), roughly half of one SPDP modification (Figure 2-16). The result was difficulty in identifying peaks as modified with one SPDP or containing two phosphate adducts. The difference in m/z for RNase A modified with one SPDP group and RNase A with two phosphate adducts was 0.18. The difference was enough to determine the identity of each peak with high resolution ESI-FTICR-MS, but was small enough to warrant the choice of another modification reagent giving a mass change that would demonstrate modification by the reagent unequivocally, and with no confusion regarding phosphate adducts.

Modification with SMPT

The modification reagent SMPT was chosen as the next reagent with which to modify RNase A. SMPT is commercially available, as is SPDP, and contains the same heterobifunctionality, introducing the possibility of further cross-linking to another functional molecule later. SMPT also satisfied the requirements of appropriate mass change to facilitate ESI-MS analysis, reactivity similar to that of NHS-acetate and SPDP and neutralization of lysine residues upon modification. The separation procedure optimized for SPDP was applied to the SMPT-modified RNase A.

RNase A was modified with SMPT and examined by ESI/FTICR-MS. These preliminary data of the unseparated derivatized protein showed series of multiply protonated $[M+zH]^{z+}$ molecular ions. The mass change to RNase A for one SMPT is an increase of 273.34 Da. Native and multiply SMPT-modified RNase A ions were identified (Figure 2-17), as well as phosphate adducts to the SMPT-modified RNase A (123,125) as normally seen in the native ESI-MS.

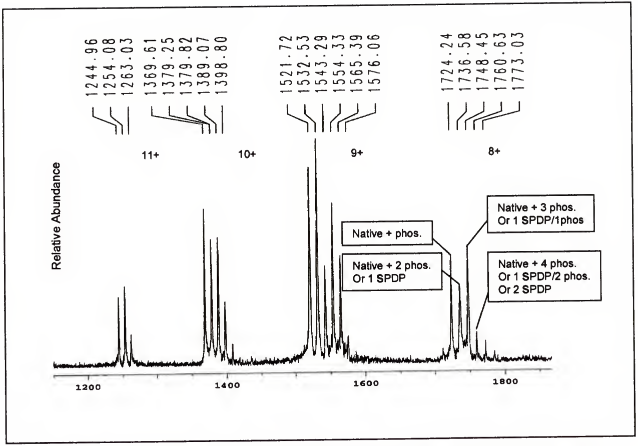


Figure 2-16. ESI/FTICR-MS of fraction from Bio-Rex 70 separation of SPDP-modified RNase A. M/z values in each charge state indicate either the addition of one SPDP group or two phosphate adducts to RNase A. M/z change for one SPDP addition to RNase A equals 24.66 at the $[M + 8H]^{8+}$ charge state, while the addition of 2 phosphate adducts results in an m/z change of 24.48 at the $[M + 8H]^{8+}$ charge state.

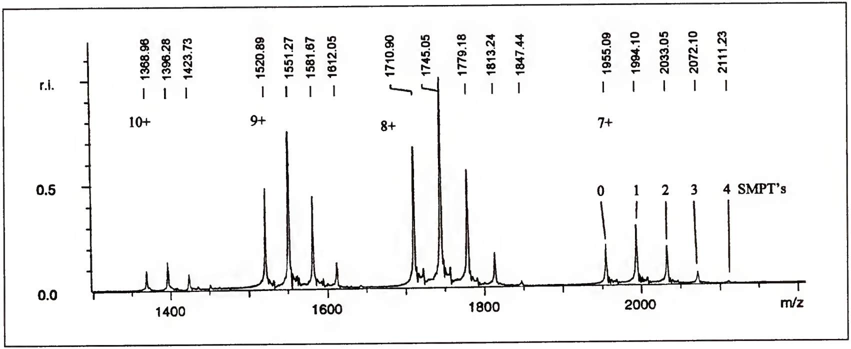


Figure 2-17. ESI/FTICR-MS multiply modified RNase A (molar ratio 1:2 RNase A to SMPT) at $[M+7H]^{7+}$, $[M+8H]^{8+}$, $[M+9H]^{9+}$and $[M+10H]^{10+}$. Peaks corresponding to 0, 1, 2, 3 and 4 SMPT modifications are indicated for the $[M+7H]^{7+}$ state. M/z values for modification at $[M+8H]^{8+}$: Modification, expected m/z $[M+8H]^{8+}$, measured m/z $[M+8H]^{8+}$; 1 SMPT, 1745.07, 1745.05; 2 SMPT, 1779.24, 1779.18; 3 SMPT, 1813.14, 1813.24; 4 SMPT, 1847.57, 1847.44.

Liquid chromatography was used to separate SMPT-modified RNase A that had been modified with different numbers of SMPT. The first separation of the modification reaction solution was performed by cationic exchange chromatography using the aforementioned Bio-Rex 70 resin. As with NHS-acetate and SPDP, the positively charged lysine residues are neutralized upon modification with SMPT. Elution on the Bio-Rex column resulted in separation of protein modified at different locations with different numbers of SMPT as well as separation from any unmodified RNase A. A typical Bio-Rex 70 separation of SMPT-modified RNase A as a result of optimized reaction and separation conditions is shown in Figure 2-18.

Highly purified RNase A was mostly singly modified with few multiple modifications at a molar ratio of 1:1 to 2:1 SMPT to RNase A. Higher concentrations of SMPT were needed with less highly purified RNase A (where the Sephadex G-50 column step was not used). It was also found that a molar concentration that was too high (e.g. 10:1 SMPT to RNase A) would over-modify highly purified RNase A, resulting in rapid elution of the entire sample from the Bio-Rex 70 column with little or no separation (data not shown).

Separated peaks were obtained using Bio-Rex 70 chromatography when the reaction buffer was at pH 8.0, as shown in Figure 2-18. With the goal of isolating increasing quantities of singly modified RNase A at a known site, a modification reaction at pH 7.0 was attempted to direct modification to the α-amino terminus group. The pKa of the α-amino terminus group has been reported as 6.6, whereas the pKa of the ε-amino group for lysine-41 has been reported as 8.6; pKa's of the other lysine ε-amino groups range from 10.6 to 11.2 (113). Lowering the pH of the reaction buffer would theoretically increase the probability of modification at α-lys-1. With a pH = 7.0 reaction, fewer resolved peaks were seen on the Bio-Rex 70 column using the same elution method (Figure 2-19). Therefore, the buffer EPPS at pH 8.0 was considered optimum for the modification reaction.

The extent of homogeneity and number of SMPT modifications for each Bio-Rex 70 peak in Figure 2-18 was examined by C-18 HPLC/ESI/QIT-MS. These results enabled semi-quantitative determination of the number of SMPT modifications in a given Bio-Rex 70 peak. HPLC/ESI/QIT-MS data for extent of homogeneity and number of SMPT modifications was acquired by selection of masses that correspond to ions of interest at expected charge states. The relative abundances of those mass selected ions were plotted individually against their elutions over time, resulting in the elution pattern for only the mass selected ion.

Separation on a C-18 HPLC column online with ESI-MS of peaks A-E, shown in Figure 2-17, indicated that proteins with various numbers of SMPT modifications were in each Bio-Rex 70 peak. One example of the HPLC/ESI/QIT-MS data (peak D, Figure 2-18) is shown in Figure 2-20. M/z ratios corresponding to ions of interest were selected and plotted individually with respect to elution time (termed mass selection chromatogram). The percentages of RNase A modified with different numbers of SMPT groups in a given peak were based on the relative abundances of certain ions under these ESI conditions. The detection sensitivities of the ions were assumed to be equal. The percentages of RNase A modified singly, doubly, etc., in each peak are summarized in Table 2-1. As expected, percentages of decreasingly modified RNase A increased with elution time, showing peak D (Figure 2-18) as having the highest percentage of singly modified RNase A. Peak E (Figure 2-18) was almost 100% unmodified RNase A. The change in number of modifications by SMPT seen in each Bio-Rex 70 fraction demonstrates the heterogeneity of each peak. The various peaks seen for each number of SMPT modifications (Figure 2-20) indicate lack of selectivity of SMPT with respect to

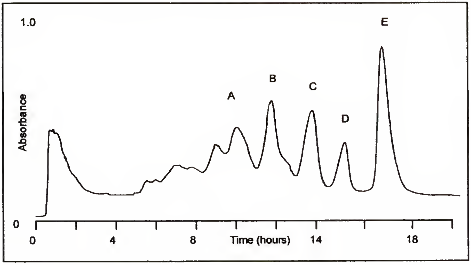


Figure 2-18. Bio-Rex 70 chromatogram of RNase A modified with SMPT. Column was eluted with an increasing exponential gradient of ammonium phosphate, 10 mM to 160 mM, at pH 7.2 and 4°C. SMPT addition to lysine residues results in neutralization of positive charge. Decreasingly modified RNase A elutes with increasing retention time (peaks A – D). Peak A has the highest relative abundance of multiply modified RNase A. Peak D has the highest relative abundance of singly modified RNase A (see Table 2-1). Unmodified RNase A (peak E) elutes last as the most highly charged species.

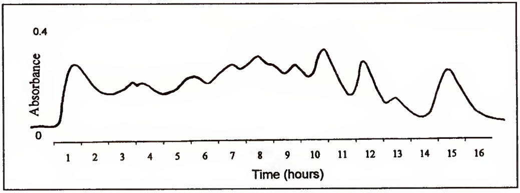


Figure 2-19. Bio-Rex 70 chromatogram of RNase A that had been modified with SMPT in a reaction solution at pH 7.0. Fewer resolved peaks were seen on the Bio-Rex 70 column using the same elution method as used for RNase A modified with SMPT at pH 8.0 (Figure 2-18).

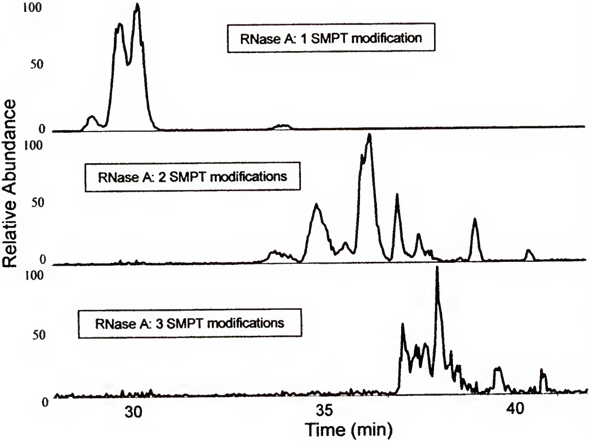


Figure 2-20. HPLC/ESI/QIT-mass chromatogram of SMPT-modified RNase A, fraction D (Figure 2-18). Mass selection for singly (top), doubly (middle) and triply (bottom) modified RNase A is shown to the right. The various peaks seen for each number of SMPT modifications indicate lack of selectivity of SMPT with respect to location of modification.

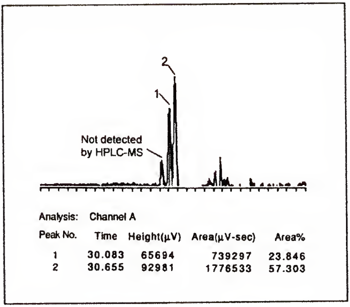
location of modification. For example, in Figure 2-20, chromatogram 2, the separation of doubly modified RNase A into many peaks indicates the SMPT modifications are at several different lysine residues. On the other hand, the singly modified appear to predominate in 2 peaks eluting at t ≈ 30 minutes from peak D (Figure 2-18).

Table 2-1. Relative abundances (%) of RNase A modified to various degrees in Bio-Rex 70 peaks[a]

Peak	0 SMPT	1 SMPT	2 SMPT	3 SMPT	4 SMPT
A	0.0	0.0	53.1	42.4	4.5
B	0.2	4.6	75.8	17.9	1.5
C	1.1	67.9	27.3	4.2	0.4
D	1.2	82.9	13.6	2.0	0.4
E	99.0	1.0	0.0	0.0	0.0

[a] Corresponds to peaks A-E in Figure 2-18.

To illustrate the possibility of obtaining protein that has been singly modified at a single, specific site for bioconjugation purposes, offline HPLC was added as a second dimension of separation after Bio-Rex 70. Offline HPLC peaks with retention times consistent with singly modified RNase A (Figure 2-21, peaks 1 and 2) were collected and examined by ESI/FTICR-MS. ESI/FTICR-MS of the sample indicated that there was only singly modified RNase A present with and without phosphate adducts (Figure 2-22). Such an example demonstrates the possibility of isolating singly modified protein for further bioconjugation. It does not, however, indicate that the singly modified sample is truly homogeneous. Mapping of the derivatized protein through proteolytic digestion and subsequent analysis must be used to demonstrate homogeneity.



Analysis: Channel A

Peak No.	Time	Height(µV)	Area(µV-sec)	Area%
1	30.083	65694	739297	23.846
2	30.655	92981	1776533	57.303

Figure 2-21. Offline HPLC chromatogram with UV-vis detection at 229 nm of SMPT-modified RNase A. Peaks shown correspond to retention time that had previously been determined to contain singly modified RNase A. Peaks 1 and 2 were collected for further examination. Both contain singly SMPT-modified RNase A.

Conclusion

RNase A was chosen for chemical modification for the purpose of developing a semi-synthetic enzyme. Chemical modification of RNase A followed by separation of reaction products was the method through which the bioconjugated system would ultimately be attained.

Original selection of the reagent with which to modify RNase A was based on its availability and the functionality it would impart to the protein upon modification. Traut's reagent is commercially available, and its reaction with RNase A resulted in terminal thiol additions to the lysine residues. However, its over-modification of RNase A was demonstrated by ESI-MS. The inability to separate the reaction products by available chromatographic methods made Traut's reagent an inappropriate choice for use in development of the bioconjugated system.

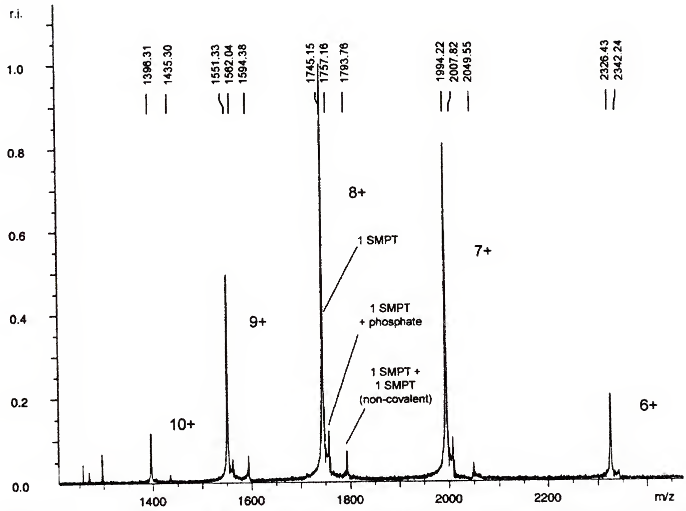


Figure 2-22. ESI/FTICR-MS of SMPT-modified RNase A from offline HPLC separation peak A (Figure 2-21). M/z 1745-[M + 8H]$^{8+}$ corresponds to single addition of SMPT to RNase A. No native or multiply modified RNase is present.

NHS-acetate was then used to modify RNase A as a control. While the product contained no functionality for subsequent functionalization, it was useful for development of separation conditions and examination of reactivity with the NHS-group. The success of the RNase A/NHS-acetate reaction system and separation of products resulted in the use of a new reagent, SPDP. SPDP possesses the same chemical reactivity with RNase A as NHS-acetate, but also containes important functionality. SPDP contains an internal disulfide that could later be exchanged with a thiol to further derivatize RNase A. However, SPDP modification was not easily identified by ESI-MS due to the mass change of RNase A once modified.

SMPT was then selected as the modification reagent to be used for the development of a potential bioconjugate. Analysis of the SMPT-modified RNase A was shown by ESI-MS to possess multiply modified RNase A. The reaction products could also be separated into fractions that contained singly modified protein by the procedure developed for acetate-modified RNase A. Mapping of the modifications at RNase A residues is the subject of the next two chapters.

Demonstrated above has been the utility of mass spectrometry for determination of the exact numbers of modifications to the protein. The evaluation of each modification reagent system was accomplished without any proteolytic digestion, and the methods illustrated for modification, separation and analysis are applicable to many chemical modification systems.

CHAPTER 3
THE MAPPING OF SMPT-MODIFIED RNASE A I: PROTEOLYTIC DIGESTION WITH TRYPSIN AND ESI/FTICR-MS ANALYSIS

Introduction

Proteolytic Digestion with Trypsin

Characterization of proteins has frequently been accomplished by cleavage of the peptide chain with proteolytic enzymes followed by analysis of the resulting peptide segments. Specificity of the proteolytic enzyme is important for cleavage of the substrate protein at desired cleavage sites. A review of useful enzymes for digestion purposes was presented by Wilkinson (142).

The serine protease trypsin is probably the most commonly used protease today. Trypsin cleaves at the C-terminal side of lysine and arginine residues based on recognition of those positively charged residues in the substrate-binding domain of trypsin. Bonds of type Lys-X and Arg-X are susceptible to cleavage. When X is proline, however, the peptide bond is resistant to cleavage. It has been reported that acidic or basic groups on the C-terminal side of lysine or arginine may reduce the rate of cleavage at that peptide bond (142). Non-specific cleavage of protein substrate may be the result of contamination of trypsin with a small amount of chymotrypsin. Chymotrypsin is a member of the serine protease family along with trypsin. Both enzymes hydrolytically cleave peptide bonds by their characteristic catalytic triad; His-57 stabilized by Asp-102 abstracts a proton from Ser-195. The difference between trypsin and chymotrypsin is found in their binding domains. Trypsin has an aspartate residue at the bottom of the binding pocket for interaction with positively charged residues. Chymotrypsin has a hydrophobic binding pocket for interaction with aromatic residue side chains. Trace chymotryptic activity in trypsin is eliminated by treatment of trypsin with L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK) that bears a hydrophobic group and results in irreversible inhibition of chymotrypsin (143).

Trypsin was selected for digestion of RNase A and SMPT-modified RNase A. It was expected that incomplete digestion of RNase A would occur and that combinations of internal peptide segments (fragments) would result as well as individual peptide segments. The primary structure of RNase A is shown in Figure 3-1 with the sites of cleavage by trypsin and the corresponding peptide segments. Digestion of native and SMPT-modified RNase A by trypsin was used to determine sites of modification by ESI/FTICR-MS. One goal of the present study was to examine the selectivity of modifications by SMPT as compared to others such as acetic and succinic anhydride (144).

Analysis of Digestion Products by ESI/FTICR-MS

Mass spectrometry was used to determine the sites of SMPT-modification by comparison of RNase A native peptide segment masses to SMPT-modified peptide segment masses. The mass analysis and detection was performed by Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. This technique enables detection of all ions for the ESI source simultaneously rather than by scanning the mass ranges as in ion trap ejection; in addition, the FTICR method omitted the step of HPLC separation of the digestion products prior to analysis in this work.

Ions formed in the electrospray source are directed to the analysis and detection cell by decreasing pressure and hexapole ion guides. The ions become trapped inside the cell by a potential applied to two plates on either end of the cell. Many different ICR cells have been reported (145). A generalized illustration of a cubic FTICR cell is shown in Figure 3-2.

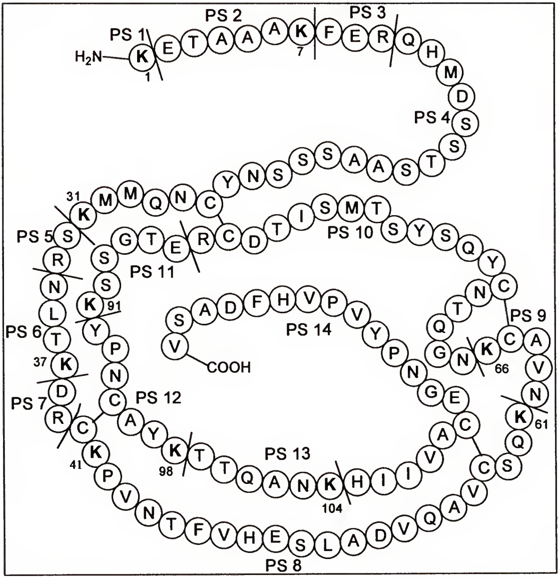

H₂N
COOH
PS 1
PS 2
PS 3
PS 4
PS 5
PS 6
PS 7
PS 8
PS 9
PS 10
PS 11
PS 12
PS 13
PS 14
1
7
31
37
41
61
66
91
98
104
K E T A A A K F E R Q H M D S S T S A A S S S N Y C N Q M M K S R N L T K D R C K P V N T F V H E S L A D V Q A V C S Q K N V A C K N G Q T N C Y Q S Y S T M S I T D C R E T G S S K Y P N C A Y K T T Q A N K H I I V A C E G N P Y V P V H F D A S V

Figure 3-1. Amino acid sequence of RNase A. Sites of cleavage by trypsin are shown. Peptide segments (PS) that result from complete cleavage are assigned. Incomplete cleavage of RNase A by trypsin can result in a combination of linked internal peptide segments, called fragments.

A magnetic field, B, applied to the cell deflects an ion of charge, q, mass, m, and velocity, v, into a circular path that is perpendicular to the magnetic field and is shown by eq 3-1.

$$\omega = q\,B/m \qquad (3\text{-}1)$$

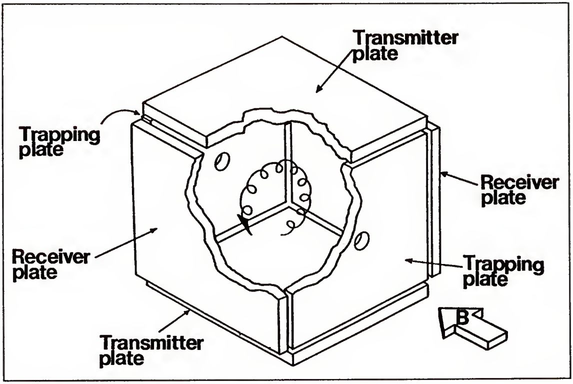


Figure 3-2. General view of an ion cyclotron resonance (ICR) cell. B represents the uniform magnetic field that is applied to the cell. A potential is applied to the trapping plates to hold the ions in the cell. The ions are deflected by the magnetic field into a circular path in the xy plane that is parallel to the magnetic field. An electronic field is applied across the transmitter plates that oscillates at an angular frequency equal or nearly equal to that of the ions of a certain q/m, causing them to cycle to a larger orbital radius while q/m is unchanged. The cycling ions induce a charge on the receiver or detector plates. The rotating charge produces an image current that is Fourier transformed into discrete signals equal to m/q or m/z.

Thus, the angular frequency, ω, is directly proportional to the charge-to-mass ratio of an ion and is independent of radius, r and velocity in the xy plane, v_{xy}. The orbital radius of the ion is related to the mass-to-charge ration by eq 3-2,

$$r = m\, v_{xy} / q\, B \qquad (3\text{-}2)$$

where v_{xy} is directly proportional to the cyclotron radius. Application of a spatially uniform electronic field oscillating at or near the cyclotron frequency of ions of a particular mass-to-charge ratio, m/q (more commonly designated m/z), results in the acceleration of ions coherently to a larger orbital radius, while ω remains unchanged. Once the orbital radius of the ions is large enough to be detected, an alternating charge is induced on the detector plates of the cell as the ions move in their cyclotron orbits. The alternating charge on the plates forms an image current induced by the ions of q/m that is separated by Fourier transformation into the sinusoidal waves that sum to give the nonsinusoidal image current (146). The sinusoidal components are then displayed as separate signals equal to the m/q ratios of the ions (145,147,148).

Marshall *et al.* (145) demonstrated that intensity of the ICR signal is proportional to the induced current on the detector plates and is independent of magnetic field strength. Intensity increases linearly with both ion cyclotron radius and ion charge but is independent of mass. Therefore, all ion intensities must be normalized according to their charges. Because ESI is a competitive process, fragmentation during ion transport is assumed to be constant across the spectrum of ESI ions observed.

Analysis of proteolytically digested RNase A and SMPT-modified RNase A by ESI/FTICR-MS allows for rapid and highly sensitive detection of peptide sequences and fragments. Comparison of the ESI/FTICR-MS spectra enables site of SMPT-modification to be determined. Factors that affect intensity of ions in the FTICR cell make quantitative analysis of the selectivity of lysine residues for SMPT modification challenging. Discussed below are the issues involved in relating concentration of a peptide to the signal intensity and relative ion abundance in the gas phase and extrapolation of the concentration to the solution-phase precursor.

Experimental Procedures

General Reagents

All reagents were reagent grade unless otherwise specified. All buffers were purchased from Sigma (St. Louis, MO). Water was purified by a Sybron/Barnsted Module Deionization System.

Trypsin Proteolytic Digest

All reagents were purchased from Sigma unless otherwise specified. The modified RNase A was dissolved in 1 mL of 0.1 M NH_4HCO_3 at pH 8.0, and guanidine hydrochloride was added to a final concentration of 6 M. A 100:1 molar excess of dithiothreitol (DTT) (Acros) to protein was then added, and the solution was incubated at 57°C for 1 hour. After cooling to room temperature, 4-vinyl pyridine was added in a 2.2:1 molar excess of 4-vinyl pyridine to DTT. The solution was then incubated at room temperature for 30 minutes. Acetic acid was added until pH <3, which stopped the alkylation. The solution was the dialyzed exhaustively against 0.1% trifluoroacetic acid (Fisher) followed by lyophilization.

The denatured protein solution was reconstituted in 1 mL of 0.1 M NH_4HCO_3, pH 8.0. Trypsin stock solution was prepared in 0.1 M NH_4HCO_3 buffer that had been acidified to pH< 3 with 3 N HCl. Five mg of trypsin was dissolved in 1 mL of the acidic buffer. A 100:1 molar ratio of RNase A to trypsin was used by adding an aliquot of trypsin stock solution to the denatured RNase A solution. The reaction mixture was allowed to incubate at 37°C for 24 hours. The digestion was stopped by lyophilization. The digested protein was reconstituted in isopropanol or 49% methanol / 49% H_2O / 2% acetic acid for examination by ESI/FTICR-MS.

Electrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

ESI/FTICR-MS data were collected by G. H. Lisa Lang and Linda S. Nichols. All ESI/FTICR mass spectrometric experiments were performed with a Bruker BioAPEX external source shielded 4.7-Tesla FT-ICR mass spectrometer (Bruker Analytical Systems, Inc., Billerica, MA) equipped with a Bruker Xmass 4.0.3 data system. A detailed description of the instrument can be found elsewhere (149,150). The electrospray source was a modified Analytica electrospray source (Analytica of Branford, Branford, CT). The stainless steel desolvation capillary was held at ground potential and 120°C. The FTICR cell pressure was 1×10^{-9} torr. The samples were sprayed at a rate of 1 μl/min. The electrospray needle (2950 V) was located at a distance ca. 1 cm from the capillary. The capillary and skimmer voltage were adjusted to values which maximized the signal intensities for the ions of interest, and typical values were 100 and 10 volts for the capillary and skimmer, respectively.

Calculations for ESI/FTICR-MS Tryptic Digest

Absolute intensities of ions were generated by using the program Xmass 4.0.3. Calculations were done using ion abundances that had been normalized by dividing the intensities by the charge of the ions (I/z) (145). The percentage of observed ions in the ESI/FTICR-MS spectra of a tryptic digest sample (native or modified RNase A) that could be assigned was calculated by summing all assigned charge-normalized ion abundances and dividing by the total sum of all normalized ion abundances, assigned and unassigned (eq 3-3). In eq 3-3, I = intensity of an ion peak, z = charge state of ion, i = 1 to N, N = total number of assigned peaks, j = 1 to M, and M = total number of peaks, assigned and unassigned.

$$\frac{\sum_{i}^{N} \left(\frac{I}{z}\right)_i}{\sum_{j}^{M} \left(\frac{I}{z}\right)_j} \times 100 = \%\ \text{peaks assigned} \qquad (3\text{-}3)$$

The estimated percent contribution of each peptide segment in the native RNase A digest was calculated according to eq 3-4, where I = intensity, z = charge state, P_n = peptide segment n, resulting from complete cleavage by trypsin, n = indices of sequence peptide (n = 1,2,3 ... 14, see Figure 3-1), f = fragment of linked peptide segments resulting from complete or incomplete cleavage by trypsin, and L = number of peptides linked in a fragment. The factor L results in the inclusion of the moles of each peptide segment being represented in the total mole sum for the spectrum, which is correlated to the total ion abundance. The first summation in the numerator of eq 3-4 includes every fragment f that contains peptide segment n. The first summation in the denominator of eq 3-4 includes every fragment f that is assigned in the spectrum, including fragments containing peptide segment n. The summations over z sum over intensities for all charge states observed for fragment f. This calculation was repeated for each digested peptide segment.

$$\frac{\sum_{f}^{\text{cont } P_n}\left(\sum_{z=1}^{z_{\max}}\left(\frac{I_f}{z}\right)\right)}{\sum_{f}^{\text{all assign}} L\left(\sum_{z=1}^{z_{\max}}\left(\frac{I_f}{z}\right)\right)} \times 100 = \begin{array}{l}\text{\% peptide n}\\ \text{in total ion population}\end{array} \qquad (3\text{-}4)$$

The estimated percentage of modification at a given lysine residue was calculated according to eq 3-5 where the definitions of all variables are the same as in eq 3-4 and where $P_n{}^*$ = peptide segment n containing a modification on its lysine residue. The first summations in the numerator and denominator over $P_n{}^*$ include every

$$\frac{\sum_{f}^{\text{cont } P_n^*}\left(\sum_{z=1}^{z_{\max}}\left(\frac{I_f}{z}\right)\right)}{\sum_{f}^{\text{cont } P_n^*}\left(\sum_{z=1}^{z_{\max}}\left(\frac{I_f}{z}\right)\right) + \sum_{f}^{\text{cont } P_n}\left(\sum_{z=1}^{z_{\max}}\left(\frac{I_f}{z}\right)\right)} \times 100 = \begin{array}{l}\text{\% modification at}\\ \text{lysine contained in}\\ \text{peptide n}\end{array} \qquad (3\text{-}5)$$

fragment that contains modified peptide segment n*. The summations over z and over P_n are the same as in eq 3-4. Eq 3-5 was used for every peptide that contains a lysine residue.

HPLC Separation of Modified RNase A Offline

Some Bio-Rex 70 separated SMPT modified RNase A fractions were further separated on a Phenomenex Jupiter 5μ C18 column (Torrance, CA) (4.6 mm i.d. x 250 mm; guard column, 4.6 mm i.d. x 30 mm, 300 Å pore). Buffer A consisted of 1% stock buffer (below), 10% acetonitrile (HPLC grade, Fisher), 89% H_2O and buffer B was 1% stock buffer, 75% acetonitrile, and 24% H_2O. The stock buffer consisted of 7.5% trifluoroacetic acid (Fisher), 50% triethanolamine (Fisher), 25% acetonitrile and 62.5% H_2O. The column was equilibrated with 5% buffer B at 1 mL/min. The elution was as follows: 0 min (A:B=95:5), 5 min (A:B=95:5), 20 min (A:B=70:30), 30 min (A:B=65:35), 40 min(A:B=25:75); 50 min (A:B=95:5). The various forms of modified RNase A were detected by their absorbance at 229 nm. The peak fractions were collected, concentrated, lyophilized and reconstituted in 49% methanol / 49% H_2O / 2% acetic acid for mass spectrometric analysis.

Results and Discussion

Tryptic Digestion of SMPT-modified RNase A

Digestion of SMPT-modified RNase A with the specific protease trypsin provides peptide segments and fragments that can be examined by ESI/FTICR-MS. Trypsin cleaves peptide bonds at the C-side of lysine and arginine residues based on recognition of those positively charged residues in the substrate-binding domain of trypsin. Derivatization of a lysine residue by SMPT neutralizes the positive charge and should eliminate the ability of trypsin to cleave at that site; this hypothesis has been supported by experimental data. Experimental data also indicates that SMPT-modification at a given lysine residue does not inhibit cleavage at the next possible site, some sites as few as 2 residues away from the SMPT modification. The changes in peptide masses and digestion pattern between native and SMPT-modified RNase A allow for modification site identification and at least partial quantification of the propensity of those sites to be modified.

Tryptic digests were performed on native and SMPT-modified RNase A. The primary sequence of RNase A is shown in Figure 3-1, noting the usual cleavage sites by trypsin and the resulting 14 peptide segment (*peptide segment* refers to one of the 14 peptides that results from complete digestion by trypsin, *fragment* refers to oligopeptides resulting from complete or incomplete digestion by trypsin and is any single peptide segment or linked peptide segments that might be observed by MS). Each digested protein sample was examined by ESI/FTICR-MS (Figures 3-3(a) and 3-3(b)). Experimental data indicated the incomplete digestion of both native and SMPT-modified RNase A. The result of incomplete digestion by trypsin was many combinations of internal peptide segments (i.e., fragments). An example of this incomplete digestion was the identification of fragment 5-7 (residues 32-39), which was seen as a linked fragment and as cleaved individual peptide segments of 5, 6 and 7 in the observed spectrum. Because of the incomplete digestion of native and modified RNase A, all possible combinations of peptide segments were calculated at all possible charge states including possible cationic adducts (H^+, Na^+, K^+, NH_4^+) to aid in peak identification in the ESI/FTICR-MS spectra. These calculations were performed using the program FragESI written by David Richardson for ESI/FTICR-MS spectra peak identification of digested proteins. Using this method (eq 3-3), approximately 75% of the total ion charge-normalized intensity in the native RNase A and 65% of the ion the total ion charge-normalized intensity in the SMPT-modified RNase A were assigned as known peptide segments or fragments. Ion peak assignments for native and SMPT-modified RNase A

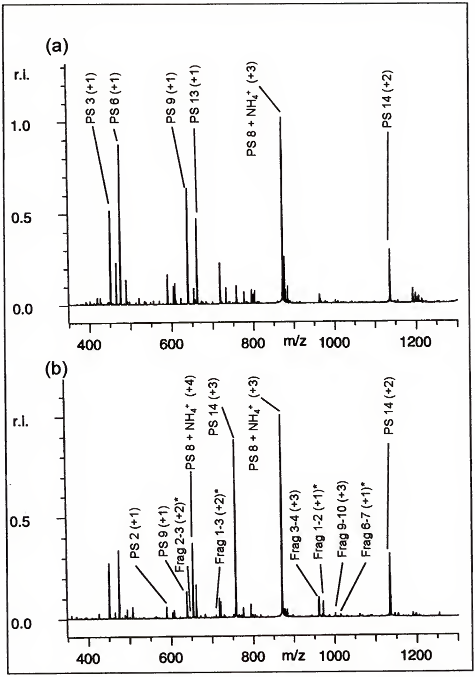


Figure 3-3. ESI/FTICR-MS spectra of tryptic digests of a) native RNase A and b) SMPT-modified RNase A. Labeled in both are assignments of ions as peptide segments (PS) or as fragments (frag) at a certain charge state, $[M + zH]^{z+}$. Fragments in b) that have an SMPT modification are denoted with *.

are listed in Tables 3-1 and 3-2, respectively. The mass change for one SMPT modification including alkylation by 4-vinyl pyridine is 268.28 Da. Spectra for the fragment containing unmodified and modified lysine 37 are shown in Figures 3-4(a) and 3-4(b).

Table 3-1:
ESI/FTICR-MS signals (m/z) detected from peptides arising from the tryptic digestion of native RNase A

Peptide or Fragment			m/z			
Number	Residues	Sequence	$[M + H]^+$	$[M + 2H]^{2+}$	$[M + 3H]^{3+}$	$[M + 4H]^{4+}$
1	1	K				
2	2–7	ETAAAK	590.2			
3	8–10	FER	451.1			
4	11–31	QHMDSSTSAASSSNYCNQMMK	489.1 - s		809.1 - s	
5	32-33	SR	301.1 - s			
6	34–37	NLTK	475.3			
7	38–39	DR	290.1			
8	40–61	CKPVNTFVHESLADVQAVCSQK		1336.5 - s	872.4 - s	668.8 - s
9	62–66	NVACK	638.9			
10	67–85	NGQTNCYQSYSTMSITDCR		1190.8	794.4	
11	86–91	ETGSSK	608.3			
12	92–98	YPNCAYK	963.9			
13	99–104	TTQANK	662.3			
14	105–124	HIIVACEGNPYVPVHFDASV		1136.4	757.9	
1–2	1–7		718.4			
5–6	32–37		718.4			
5–7	32–39				329.7	
11–13	86–104			1096.8	756.3 - s	
4–5	11–33			1336.5 - s	891.4 - s	
9–10	62–85			1501.1	1008.1 - s	
8–9	40–66				1086.3 - s	
11–14	86–124				1491.1 - s	1118.6 - s
5–8	32–61				1204.1 - s	
3–4	8–31			1441.6 - s	962.1 - s	721.6 - s
11–12	86–98			776.5		
7–8	38–61				961.7 - s	
1–7	1–39				1513.8 - s	

s indicates salts that are seen as adducts to the peptide segment and are subtracted from the m/z listed to give the m/z of the peptide segment ion.

Table 3-2: ESI/FTICR-MS signals (m/z) detected from peptides arising from the tryptic digestion of SMPT-modified RNase A.

Peptide or Fragment			m/z			
Number	Residues	Sequence	[M + H]$^+$	[M + 2H]$^{2+}$	[M + 3H]$^{3+}$	[M + 4H]$^{4+}$
1	1	K				
2	2–7	ETAAAK	590.1			
3	8–10	FER	451.2			
4	11–31	QHMDSSTSAASSSNYCNQMMK			809.9 - s	
5	32-33	SR	262.1			
6	34–37	NLTK	475.1			
7	38–39	DR	290.1			
8	40–61	CKPVNTFVHESLADVQAVCSQK			871.3 - s	653.8 - s
9	62–66	NVACK	639.1			
10	67–85	NGQTNCYQSYSTMSITDCR		1190.4	794.3	596
11	86–91	ETGSSK	608.1	323.3 - s		
12	92–98	YPNCAYK		482.0		
13	99–104	TTQANK	662.1	330.1		
14	105–124	HIIVACEGNPYVPVHFDASV		1135.7	757.6	568.1
1–2*	1–7	K*ETAAAK	986.9	494.1		
1–2	1–7	KETAAAK	718.1	359.6		
5–6	32–37	SRNLTK	718.1	359.6		
11–13*	86–104	ETGSSK*YPNCAYK*TTQANK				616.8
4–5*	11–33	QHMDSSTSAASSSNYCNQMMK*SR				731.7
9–10*	62–85	NVACK*NGQTNCYQSYSTMSITDCR			1090.7	818.2
9–10	62–85	NVACKNGQTNCYQSYSTMSITDCR			1001.1	
3–4	8–31	FERQHMDSSTSAASSSNYCNQMMK		1440.2 - s	960.9 - s	
11–12	86–98	ETGSSKYPNCAYK		776.4		
11–12*	86–98	ETGSSK*YPNCAYK		910.8		
5–7*	32–39	SRNLTK*DK		629.6		
5–7	32–39	SRNLTKDK			329.7	
6–7*	34–39	NLTK*DR	1014.7	508.1		
2–3	2–10	ETAAAKFER		530.0 - s		
2–3*	2–10	ETAAAK*FER		646.04		
12–13	92–104	YPNCAYKTTQANK			563.4 - s	
12–13*	92–104	YPNCAYK*TTQANK			625.7	
1–3*	1–10	K*ETAAAK*FER		710.0		
13–14*	99–124	TTQANK*HIIVACEGNPYVPVHFDASV			1061.4	

s indicates salts that are seen as adducts to the peptide segment and are subtracted from the m/z listed to give the m/z of the peptide segment ion.

* indicates modification at lysine residue. Peptide segments with 2 * indicate that modification is at one or the other lysine, not both.

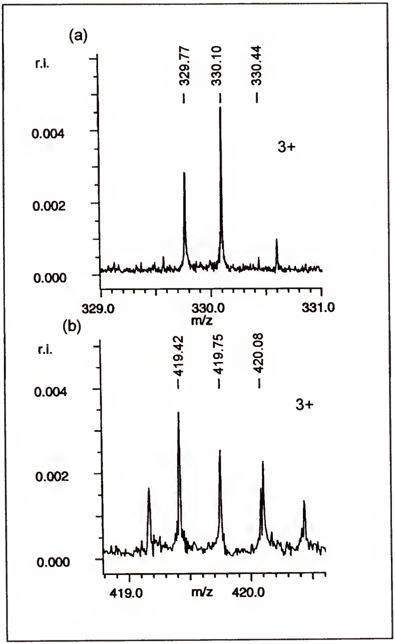


Figure 3-4. RNase A fragment 5-7 (residues 32-39) resulting from tryptic digest in a) is unmodified at m/z 329.77-$[M + 3H]^{3+}$ and in b) is singly modified with SMPT at m/z 419.42-$[M + 3H]^{3+}$. The mass difference between the ions in a) and b) is equal to 268, one SMPT modification + ethyl pyridine. Ethyl pyridine is added during denaturation of the protein prior to digestion.

Quantification of the absolute abundances of ions corresponding to modified peptides was attempted in order to determine the propensity of a given residue to be modified. It has been shown by Marshall *et al.* (145) that ICR signal increases linearly with ion charge but is independent of mass, such that ICR is increasingly sensitive for multiply charged ions. Therefore, ion intensities were normalized by dividing by their charge states. Summation of the absolute intensities for ions of interest that had been normalized according to their charge state was used as a method for the correlation of absolute abundance (peak area) of an ion to the ion concentration (ion population) and thus, SMPT modification on that peptide segment (145,151,152). In order to make such a correlation, assigned ions in the native RNase A tryptic digest were normalized and summed to verify their percent in the overall assigned ion population. (See eq 3-4 in experimental section). It was found that certain assigned ions (corresponding to certain peptide segments) represented different percentages of the total assigned ion population. Considering that the number of moles of each peptide peptide segment (1, 2, 3, ...14) is the same in the sample, the different percentages of ions (corresponding to certain peptide segments) indicated that peptide segments were being detected with varying sensitivities (Figure 3-5). If the MS method were non-discriminating, each segment would have a percent abundance of 1/14 x 100 (7.1%). Conditions that occur in the electrospray source, either in the solution droplets or in the gas phase, may give rise to interactions that result in variable detection of peptide segments. Interactions may include neutralization of charge by charge transfer from one peptide segment or fragment to an acceptor, resulting in a decreased overall charge of some ions and possibly neutralization, which leads to decreased detection of a peptide segment. Such charge reduction is favored by high charge repulsion in small, multiply charged peptide segments or fragments. Detection sensitivity may also be related to the chemical nature of the residues on each peptide segment (153-155). These factors could influence the

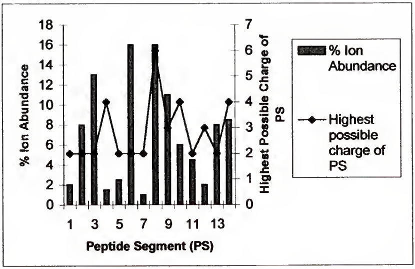


Figure 3-5. Percent ion abundance of each peptide segment in native tryptic digest (calculated according to eq 3-3) and the highest possible charge state of the peptide segment. There appears to be no equality of % ion abundance between the normalized ion intensities nor is there correlation between % ion abundance of a peptide segment and its number of basic residues.

detection sensitivity of a peptide segment ion and thus, its ion population in the protein digest spectrum. The results shown in Figure 3-5 also indicated no clear correlation between the number of basic residues in a peptide segment and its sensitivity to detection as has been previously suggested (156,157).

Peptide segment 8 (residues 40-61) has the highest number of basic residues, 5, and was also found to have the highest percentage of ion abundance in the total assigned ion population. The basic residues, lysine, arginine, pyridylethylcysteine and histidine (ES conditions) provide sites for positive charge on the ion. The increased number of basic residues, as in peptide segment 8, may increase the propensity of the ion to maintain positive charge and be detected. Therefore, it is presumed that the peptide segments with lower ion intensities have decreased percentage of the total assigned ion abundance because they are either part of the unassigned ion population or were chemically neutralized in the source.

The lack of an unbiased distribution for assigned ions in the native RNase A sample illustrates the care that should be taken when extracting quantitative data from gas-phase ion populations and using them to deduce the composition of the solution phase.

The propensity of each lysine residue to be modified by SMPT was calculated based on the information presented above. Ion abundances used to identify sites of modification, or lack thereof, were compared to their unmodified counterparts in the native digest spectrum (see eq 3-5 in experimental section). Such ion abundances were used in calculations if intensities were similar in both unmodified and unmodified spectra. As an example, peptide segment 8 contains lysine 41 and was found to be detected with greater intensity in the native digest spectrum than other peptide segments. It was concluded that SMPT modification does not occur significantly at lysine 41 because unmodified peptide segment 8 was found to be highly sensitive to detection in the SMPT-modified digest spectrum, yet no modified fragment with peptide segment 8 was found. If there was little detection of unmodified peptide segment 8 in the SMPT-modified digest spectrum, it could not be concluded that modification had not occurred at lysine 41 because the SMPT-modified peptide segment 8 may have been neutralized as described above. This exercise of comparison of sensitivities in the native and modified digest spectra was performed for each assigned peptide segment. The percent of a given assigned ion (corresponding to a modified peptide segment) in the total population of assigned ions containing that same peptide segment (modified and unmodified) was calculated. The results are shown in Table 3-3.

Table 3-3. Percentage of SMPT-modification at lysine residues [a]

Lysine	< 1%	1 - 10 %	10 - 50 %
1			x
7		x	
31		x	
37			x
41	x		
61	x		
66		x	
91		x	
98		x	
104	x		

[a] In the digested modified RNase A, x % of lysine listed has been shown to be modified with SMPT.

Apparent selectivity of SMPT for lysine residues differed from the acetic and succinic anhydride modifications reported in the literature (144,158-160). Glocker *et al.* has shown lysines 1 and 7 to have a propensity for modification by acetic and succinic anhydride as well as active site lysine 41 (144). Our experimental data suggest no significant modification of lysine 41 by SMPT occurs under our reaction conditions. Modification at lysine 7 did not differ in selectivity by SMPT from other lysines such as 31, 66, 91 and 98. Predominant modification by SMPT occurs at lysines 1 and 37. In the 3-dimensional structure of RNase A all lysines are accessible to the solvent, and lysines 1 and 37 are on the same side of the protein. Their similar location in the tertiary structure may be related to their increased propensity for modification. It is likely that modification at either residue would result in a similar change in the dipole moment of the protein, making its separation by cationic exchange chromatography difficult.

Tryptic Digestion of Singly SMPT Modified RNase A

The issues discussed for analysis of SMPT-modified RNase A led to the digestion of RNase A that had previously been shown to be singly modified. In chapter 2, offline HPLC was used as a second dimension of separation for SMPT-modified RNase A, and ESI/FTICR-MS was used to demonstrate the isolation of singly modified RNase A with no native protein present (Figure 2-22). Tryptic digestion of the two-dimensionally separated, singly SMPT-modified RNase A was performed. Experimental results were consistent with the heterogeneity of modification site in each fraction despite the increased chromatographic separation inherent in a two-dimensional method. Such an example demonstrates the difficulty of targeting single specific residues in a protein containing many reactive sites with a non-specific modification reagent.

Conclusion

ESI/FTICR-MS has been used to directly detect the extent of modification of RNase A by SMPT. Analysis of native and SMPT-modified RNase A tryptic digests by ESI/FTICR-MS resulted in the identification of the sites of modification. Semi-quantitative results of the reactivity of certain lysine residues toward the coupling reagent SMPT are possible but must be examined with caution since sensitivities of different peptide segments to detection change based on the chemical nature of the peptide and reactions in the electrospray source. Two sites (lysines 1 and 37) are highly reactive, while three sites (lysines 41, 61 and 104) appear to be unreactive under the conditions used. Quantitative results would require the use of internal standards of each peptide segment and potential combinations of peptide segments, both modified and unmodified. However, without internal standards, deduced propensities of modification at a given site will only be semi-quantitative.

CHAPTER 4
THE MAPPING OF SMPT-MODIFIED RNASE A II: PROTEOLYTIC DIGESTION WITH SUBTILISIN WITH HPLC/ESI/QIT-MS AND MS^n ANALYSIS

Introduction

Complete characterization of SMPT-modified RNase A requires the use of proteolytic digestion of the peptide chain into smaller peptide segments followed by analysis. Analysis of SMPT-modified RNase A using the serine protease trypsin and direct analysis by ESI/FTICR-MS was described in Chapter 3. Qualitative results demonstrated higher propensity for modification by SMPT at Lys-1 and Lys-37 than at other lysine residues. SMPT modification at Lys-1, and to a lesser degree Lys-7, has been investigated further by proteolytic cleavage of SMPT-modified RNase A with a different enzyme, the bacterial protease subtilisin.

The non-specific protease subtilisin cleaves RNase A into two polypeptides, the S-peptide (residues 1-20) and the S-protein (residues 21-124) (see Figure 4-1(a)). The S-peptide and S-protein tightly associate to form RNase S, which maintains the original RNase A conformation under certain conditions and retains high enzymatic activity (161,162). Residues 3-13 of the S-peptide form an α-helix when in the RNase S complex, as seen in native RNase A (see Figure 4-1 (b)). Removal of residues 16-20 from the S-peptide results in a truncated version of the peptide that retains α-helix conformation in the RNase S complex (163). Identical crystal structures of RNase A, RNase S and RNase S with the truncated S-peptide (164), along with retention of activity in RNase S, illustrate a classic example of the importance of noncovalent interactions on enzyme tertiary structure (165).

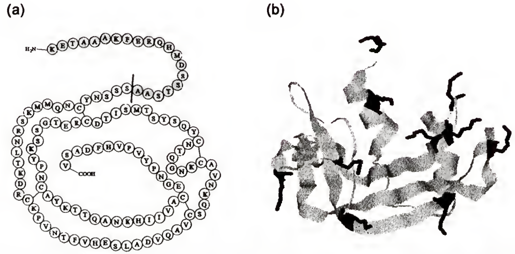


Figure 4-1. Ribonuclease A. a) Amino acid sequence showing primary site of cleavage by subtilisin into two polypeptides, S-peptide (residues 1-20) on the N-terminal side of the line and S-protein (residues 21-124) on the C-terminal side. b) Ribbon structure showing α-helical structure of the S-peptide region when covalently bound as in RNase A or noncovalently bound as in RNase S. The α-helical conformation of the S-peptide is maintained when residues 16-20 are removed, the truncated version of S-peptide, and it is complexed to the S-protein.

Many studies of RNase S-peptide and S-protein complex have been presented. Schreier and Baldwin reported on the investigation of unfolding of the RNase S complex by monitoring tritium exchange kinetic differences between S-peptide when free and when complexed to the S-protein (166). Connelly *et al.* used titration calorimetry to study the thermodynamics of the S-protein/S-peptide interactions and those of S-protein with S-peptide mutants for the evaluation of hydrophobic interactions in the stabilization of proteins (165). Alexandrescu *et al.* reported entropy changes upon folding of the S-protein/S-peptide complex by determination of ^{15}N relaxation parameters (167). Electrospray ionization mass spectrometry was used to study the thermal denaturation of RNase S to demonstrate that solution properties of noncovalent complexes can be obtained by such a gas-phase study (168,169).

The use of subtilisin for cleavage of SMPT-modified RNase A allows for identification of modification to the Lys-1 or Lys-7 position on the S-peptide. Separation and analysis of the S-peptide and S-protein (native and modified) is accomplished by HPLC/ESI/QIT-MS. MS^n has been used to examine SMPT-modification at α-Lys-l, ε-Lys-l and ε-Lys-7. HPLC online with ESI/QIT-MS separates unmodified and modified S-peptides and S-proteins, but also separates modified S-peptides by site of modification. Subsequent online analysis by ESI/QIT-MS and MS^n provides data for identification of the modified lysine residue. A diagram of the HPLC inlet to the ESI/QIT-MS system is shown in Figure 4-2.

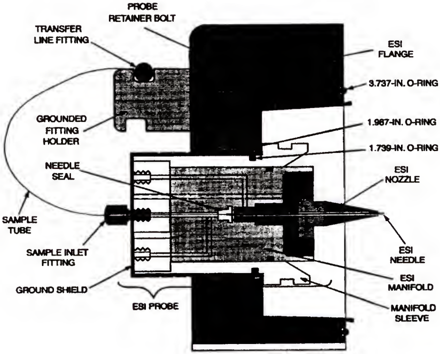


Figure 4-2. HPLC/ESI/QIT-MS inlet system (170).

Offline HPLC has also been used for the isolation of SMPT-modified RNase A. HPLC fractions that were shown to contain singly modified RNase A were digested with subtilisin followed by HPLC/ESI/QIT-MS. Results of this experiment introduced the possibility that some fraction of the covalently bound modifying reagent was being cleaved by subtilisin.

It has been noted in the literature that subtilisin, more specifically subtilisin-Carlsberg, cleaves most specifically between residues 20 and 21, but also cleaves between residues 21 and 22 to a significantly lesser degree (168,171,172). A study by Neumann *et al.*(172) reported 85-90% of the S-protein product was RNase 21-124 with 10-15% contamination with the shortened form, RNase 22-124.

Evidence for cleavage of RNase A between residues other than 20-21 and 21-22 has not been reported in the literature. In this work, the semi-quantitative examination of the products of cleavage of RNase A by subtilisin-Carlsberg was investigated by using HPLC/ESI-MS. The effect of covalent modification by SMPT at lysine 1 or lysine 7 or both on the specificity of subtilisin is also examined.

Experimental Procedures

General Reagents

All reagents were reagent grade unless otherwise specified. All buffers were purchased from Sigma (St. Louis, MO). Water was purified by a Sybron/Barnsted Module Deionization System.

Subtilisin Proteolytic Digest

Purified native RNase A or SMPT-modified RNase A was dissolved in 0.1 M NH_4HCO_3 at pH 8.0 to a volume of 500 μL. A stock solution of subtilisin (Carlsberg, Sigma) was prepared by dissolving 5 mg of the protease in 100 mL of 0.1 M NH_4HCO_3 at pH 8.0. Ten μL of subtilisin stock was added to the 500 μL RNase A solution. The mixtures were allowed to incubate for 24 hours at 4°C. Digestion was stopped by lyophilization and the lyophilates were reconstituted in 49% methanol / 49% H_2O / 2% acetic acid for mass spectrometric analysis.

HPLC/ESI/QIT-MS and MS^n

HPLC/ESI/QIT-MS and MS^n data were collected by Jodie V. Johnson. HPLC/ESI-MS and MS^n data were acquired by using a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnegan MAT, San Jose, CA) operated in the electrospray ionization (ESI) mode. Typical operating parameters were nitrogen sheath and auxiliary gases of 70 and 10 (unitless), respectively, heated capillary temperature of 235°C, and ion spray voltage of 3.5 kV. The source collision-induced dissociation (SCID) was set between 10-20% maximum for declustering. MS and MS^n data were acquired with the normal m/z 50-2000 range, as well as with the extended m/z range (up to m/z 4000) offered with the LCQ's advanced scan function software. Parent ion isolation was typically 5 u wide with the CID energy set to 35 or 50% maximum for $z = +2$ ions and 65% for $z = +1$ ions. A higher resolution scan (Zoom scan) was obtained only in the normal m/z 50-2000 range.

The mobile phase was introduced by a Beckman Instruments (Fullerton, CA) System Gold model 126 pump. A binary HPLC mobile phase system was used: buffer A consisted of 1% stock buffer (below), 10% acetonitrile (HPLC grade, Fisher), and 89% H_2O, while buffer B was 1% stock buffer, 75% acetonitrile, and 24% H_2O. The stock buffer consisted of 7.5% trifluoroacetic acid (Fisher), 50% triethanolamine (Fisher), 25% acetonitrile and 62.5% H_2O. Chromatographic separation was achieved with a Phenomenex Jupiter 5μ C18 column (Torrance, CA) (4.6 mm i.d. x 250 mm; guard column, 4.6 mm i.d. x 30 mm, 300 Å pore). The elution gradient was as follows: 0 min (A:B=95:5), 5 min (A:B=95:5), 20 min (A:B=70:30), 30 min (A:B=65:35), 40 min(A:B=25:75); 50 min (A:B=95:5). The column effluent was split prior to the ESI source with approximately 70-80% of the effluent going to waste. Relative amounts of the various peptides and proteins were determined by integration of peak area for the HPLC mass chromatogram ions of interest.

HPLC Separation of Modified RNase A Offline

Some Bio-Rex 70 separated SMPT modified RNase A fractions were further separated on a Phenomenex Jupiter 5μ C18 column (Torrance, CA) (4.6 mm i.d. x 250 mm; guard column, 4.6 mm i.d. x 30 mm, 300 Å pore). Buffer A consisted of 1% stock buffer (below), 10% acetonitrile (HPLC grade, Fisher), 89% H_2O and buffer B was 1% stock buffer, 75% acetonitrile, and 24% H_2O. The stock buffer consisted of 7.5% trifluoroacetic acid (Fisher), 50% triethanolamine (Fisher), 25% acetonitrile and 62.5% H_2O. The column was equilibrated with 5% buffer B at 1 mL/min. The elution was as follows: 0 min (A:B=95:5), 5 min (A:B=95:5), 20 min (A:B=70:30), 30 min (A:B=65:35), 40 min(A:B=25:75); 50 min (A:B=95:5). The various forms of modified RNase A were detected by their absorbance at 229 nm. The peak fractions were collected, concentrated, lyophilized and reconstituted in 49% methanol / 49% H_2O / 2% acetic acid for mass spectrometric analysis.

Results and Discussion

Cleavage Specificity of Subtilisin for RNase A

The cleavage of subtilisin for the substrate RNase A at various peptide bonds was quantified by HPLC/ESI-MS. Ions were mass-selected that corresponded to the m/z ratio for $[M + H]^{+1}$ and $[M + 2H]^{+2}$ ions of the S-peptide, such that their abundances could be integrated and summed. Because the ions being compared are the same charge states and have similar chemical structure, their relative abundances were compared without using an internal standard. Mass selection for ions of unmodified S-peptides of residues 1-16, 1-17, 1-18, 1-19, 1-20, 1-21 and 1-22 were chosen for their close proximity to the main site of cleavage. The amino acid sequence of RNase A with the primary and secondary sites of cleavage by subtilisin is shown in Figure 4-3. The HPLC mass chromatograms for each of these S-peptide ions are shown in Figure 4-4.

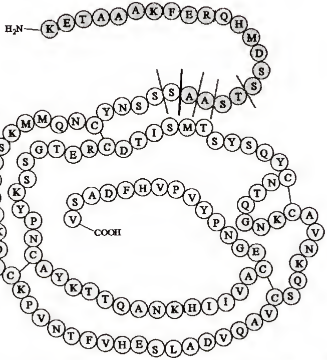


Figure 4-3. Amino acid sequence of RNase A showing sites of cleavage by subtilisin. Black is the primary cleavage site and gray represents secondary cleavage sites.

Comparison of their relative intensities revealed that 86.0 ± 3.2 % represented ions assigned as S-peptide residues 1-20, demonstrating cleavage by subtilisin between these residues. Cleavage distributions for other peptide bonds are given in Figure 4-5. The cleavages between residues 19-20, 18-19 and 16-17 represented averages of 3.0 ± 1.6 %, 7.0 ± 3.3 % and 2.1 ± 1.2 % of the S-peptide cleavage products, respectively. The production of these latter three peptides had not been reported previously in the literature. The distribution of ions includes those ions assigned to contain chemical

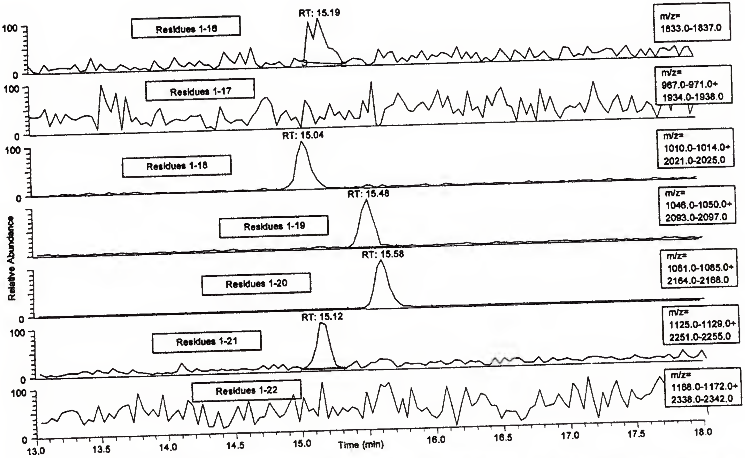


Figure 4-4. HPLC/ESI-mass chromatograms of S-peptides of varying lengths. M/z selected for each peptide of interest is shown to the right. Each peak indicates the presence of the ion that has been selected and its elution pattern from the HPLC column. Elution patterns for S-peptides are shown for residues 1-16 through 1-22.

changes to the peptides that occur under these reaction conditions such as esterification of the C-terminus end to the methyl ester or adducts of trifluoroacetic acid.

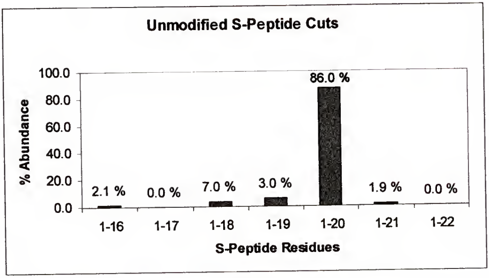


Figure 4-5. Percent relative ion abundance of S-peptides of lengths 1-16 through 1-22, indicating the propensity of the peptide bond to be cleaved by subtilisin.

Retention times of the S-peptides were found to be directly related to the chemical nature of the C-terminus residue remaining after digestion with subtilisin (see Table 4-1). The retention times on the reverse-phase column were shorter for polar C-terminus residues. The longest retention time was for S-peptide residues 1-20, which has two nonpolar alanine residues at the C-terminus end.

Modification of RNase A SMPT at lysine 1 or lysine 7, or both, that was isolated from randomly modified RNase A was used to examine the specificity of subtilisin on a chemically altered RNase A. Ions $[M + H]^{1+}$ and $[M + 2H]^{2+}$ were mass-selected from the HPLC elution gradient for S-peptides that had been modified with SMPT at one or two lysine residues. Ions that corresponded to a single modification on the S-peptide that had been cleaved at the various cleavage sites were compared for their relative

Table 4-1. Retention times of S-peptides during reverse-phase HPLC in relation to the nature of residues remaining after cleavage by subtilisin.

S-Peptide Residues	C-terminal Sequence	Reverse Phase Retention Time (min)	Chemical Nature and Number of Residues Added[a]
1–16	–SS-OH	15.19	—
1–17	–SST-OH	not detected	T, polar
1–18	–SSTS-OH	15.04	S, polar
1–19	–SSTSA-OH	15.48	A, nonpolar
1–20	–SSTSAA-OH	15.58	A, nonpolar
1–21	–SSTSAAS-OH	15.12	S, polar
1–22	–SSTSSAASS-OH	not detected	S, polar

[a] Residues represented by abbreviations: T, threonine; S, serine; A, alanine.

abundances. The SMPT-modification neutralizes the lysine residue and reduces the highest potential charge state of the peptide. Because the distribution of sensitivity to detection was generally consistent within each group of SMPT additions (i.e. 0, 1, and 2), comparison of the relative intensities of the same charge states were used without internal standards. The mass chromatograms with mass selections for singly modified S-peptides are shown in Figure 4-6. Separation of three products in each mass chromatogram in Figure 4-6 results from the different locations where SMPT modification can occur: α-lysine 1, ε-lysine 1 and ε-lysine 7 (see below).

Similar mass selection was performed for $[M + H]^{1+}$ and $[M + 2H]^{2+}$ ions that were modified at both lysines 1 and 7 (Data not shown). The data for cleavage by subtilisin of the singly and doubly SMPT-modified S-peptides is shown in Table 4-2. Each modification appears to have a negligible effect on the ability of subtilisin to cleave RNase A at any of the observed cleavage sites. Constant specificity is presumed to occur due to the distance between the modification(s) and the cleavage sites, a minimum of eight residues. It is possible that modification closer to the site of cleavage

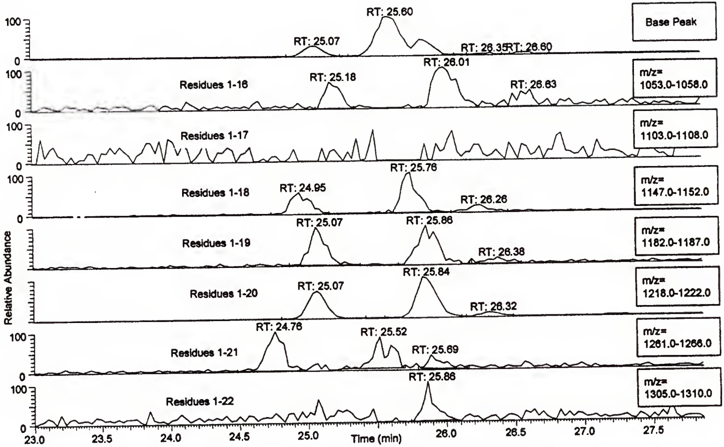


Figure 4-6. HPLC/ESI-mass chromatograms of singly SMPT-modified S-peptides of varying lengths. M/z selected for each modified peptide of interest is shown to the right. Each peak indicates the presence of the ion that has been selected and its elution pattern from the HPLC column. Elution patterns for singly SMPT-modified S-peptides are shown for residues 1-16 through 1-22.

or with a different modification reagent would alter the specificity of subtilisin for the RNase A substrate.

Table 4-2. Average percentages of S-peptides resulting from cleavage between different residues of native and SMPT-modified RNase A.[a]

Residues	0 SMPT	1 SMPT	2 SMPT[b]
1-16	2.1 ± 1.2 %	2.3 ± 0.5 %	undetected
1-17	undetected	undetected	undetected
1-18	7.0 ± 3.3 %	5.0 ± 2.3 %	4 %
1-19	3.0 ± 1.6%	3.6 ± 0.6 %	4 %
1-20	86.0 ± 3.2%	85.5 ± 5.1 %	89 %
1-21	1.9 ± 1.5%	3.4 ± 1.7 %	3 %
1-22	undetected	0.2 ± 0.1 %	undetected

[a] Average relative uncertainty is 48% for zero SMPT addition, and 28% for one SMPT addition.
[b] Data collected for one sample only.

Another factor that presented difficulty in the quantification of subtilisin specificity was the presence of two ions, m/z 1197-$[M + 2H]^{2+}$ and m/z 1090-$[M + 2H]^{2+}$, that were similar in m/z to the unmodified S-peptides and had similar HPLC elution patterns. CID of these ions resulted in fragmentation patterns that indicated their identities as chemically altered forms of the unmodified S-peptide. The identity of the ion m/z 1090 – $[M + 2H]^{2+}$ has been identified as S-peptide, residues 1-20, with a methyl ester at the C-terminus end rather than a carboxylic acid. This chemical change to the S-peptide following digestion occurred possibly when the digestion products were reconstituted in 49% methanol / 49% H_2O / 2% acetic acid. The ion m/z 1090 –$[M + 2H]^{2+}$ was, therefore, included in the total ion abundance representing the S-peptide cleaved between residues 20 and 21.

The identity of the chemical change to the S-peptide during or following digestion that resulted in the presence of the ion m/z 1197 –$[M + 2H]^{2+}$ was assigned as two adducts of trifluoroacetic acid. The presence of this ion represented a significant percentage of the total S-peptide seen in the spectra. The ion m/z 1197 –$[M + 2H]^{2+}$ was included in the total ion abundance representing the S-peptide cleaved between residues 20 and 21.

The results of the examination of specificity of subtilisin for RNase A substrate indicate the wide variation in cleavage sites and specificity for those sites under controlled reaction conditions. Many researchers have made use of the S-peptide/S-protein complex as a method for introduction of a synthetic group into the enzyme, usually by placement of a mutation by solid-phase synthesis or chemical modification of the S-peptide (164,173,174). In light of the results above, the preparation of RNase S and its components must be carefully considered before assuming purity.

Subtilisin Digests of Native and SMPT-Modified RNase A Following Bio-Rex 70 Chromatography

Digestion of modified RNase A with subtilisin was used as a complement to the modification site information obtained from tryptic digestion. A detailed examination of modifications at Lys-7 and the ε- and α-amino positions on Lys-1 was carried out. All CID data on the S-peptide were collected through fragmentation of the predominant unmodified and SMPT-modified S-peptides, m/z 1083-$[M+2H]^{2+}$, m/z 1220-$[M+2H]^{2+}$, respectively (residues 1-20).

Fractions of SMPT-modified protein from the Bio-Rex 70 column were digested by subtilisin followed by separation and examination by HPLC/ESI/QIT-MS. A variety of combinations of unmodified S-peptide, m/z 1083- $[M+2H]^{2+}$, singly modified S-peptide, m/z 1220-$[M + 2H]^{2+}$, unmodified S-protein, m/z 2882-$[M+4H]^{4+}$, singly modified S-protein, m/z 2954-$[M + 4H]^{4+}$, as well as multiply modified peptides were found in Bio-

Rex 70 fractions (see an example in Figure 4-7). The presence of these various ions demonstrates the heterogeneity in a given Bio-Rex 70 peak in terms of number of SMPT modifications and site of modification. Charge states were obtained under high-resolution detection for ions of interest to confirm peptide assignment.

A characteristic pattern of elution was observed from the HPLC/ESI/QIT-MS, showing three peaks with the same m/z that correspond to singly modified S-peptide, m/z 1220- $[M+2H]^{2+}$ (Figure 4-8). The isotopic pattern that confirms the $[M+2H]^{2+}$ state for the singly modified S-peptide is shown in Figure 4-9.

The reproducible HPLC elution pattern of singly modified S-peptide, m/z1220- $[M+2H]^{2+}$ peaks would logically correspond to the three possible sites of modification on the S-peptide: α-lys-1, ε-lys-1 and lys-7 (Figure 4-10). The technique of MS^n was used to determine the collision-induced dissociation (CID) patterns of the unmodified S-peptide ion, m/z 1083- $[M+2H]^{2+}$, and modified S-peptide ions, m/z 1220- $[M+2H]^{2+}$, to obtain sequence information about the SMPT modification site (see Figure 4-11(a) and 4-11(b)).

A characteristic CID pattern was seen for the unmodified S-peptide, m/z 1083- $[M+2H]^{2+}$. CID of the singly modified S-peptide ions at m/z 1220- $[M+2H]^{2+}$ resulted in many daughter ions (Tables 4-3 – 4-6). Ions which are indicative of the modification site in each m/z 1220-$[M+2H]^{2+}$ HPLC/ESI mass spectrum were mass selected and plotted with respect to elution time (see Figure 4-12). Peaks A and B of Figure 4-12 correspond to modification at lys-1 and peak C corresponds to modification at lys-7.

It would be difficult to determine which peak (A or B) corresponds to modification at α-lys-1 or ε-lys-1 because the CID fragmentation patterns could possibly be identical. One clue, however, to the site of lys-1 modification was found. CID fragmentation appears to be negligible at peptide bonds connected to the modified residue in peak B.

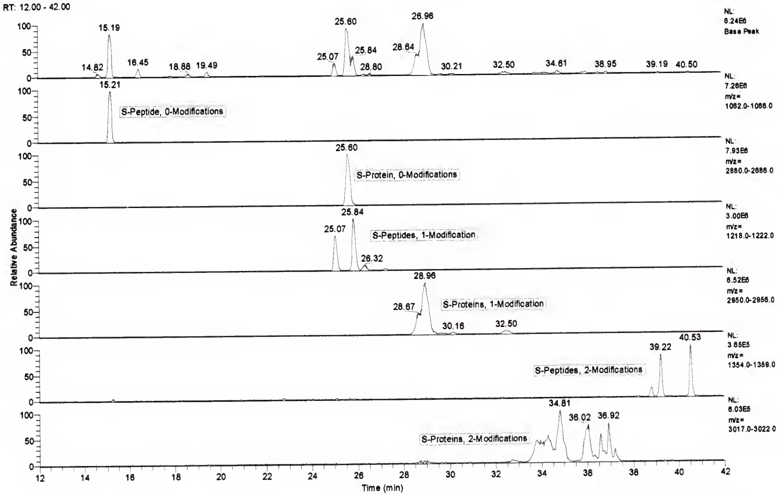


Figure 4-7. HPLC/ESI-mass chromatograms of SMPT-modified RNase A. A fraction collected from Bio-Rex 70 separation was digested with subtilisin. Mass chromatograms for unmodified, singly modified and doubly modified S-peptide and S-protein are shown with mass selections to the right. The presence of all ions selected demonstrates the heterogeneity of the Bio-Rex 70 fraction with regard to number and location of SMPT modifications.

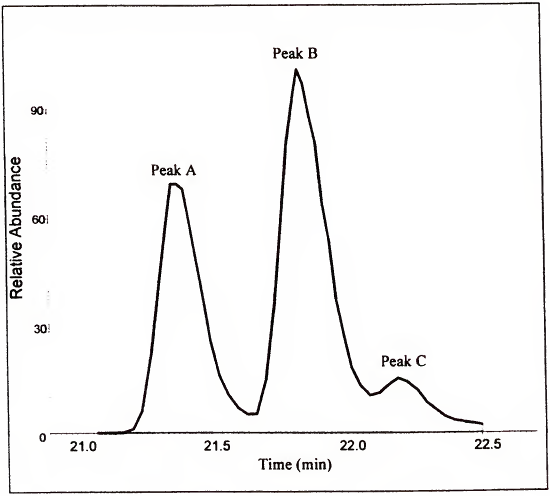


Figure 4-8. HPLC/ESI-mass chromatogram of ions m/z 1220-[M + 2H]$^{2+}$ that corresponds to singly modified S-peptide. All three peaks have the same m/z, indicating separation during HPLC elution based on differing chemical structure.

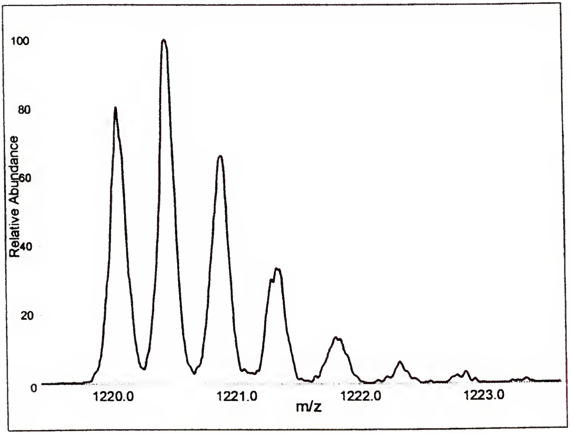


Figure 4-9. High-resolution scan of m/z 1220-$[M + 2H]^{2+}$ peak A of Figure 4-8. Isotopic pattern of the ion shows the ^{12}C peak with single additions of ^{13}C. Other isotopes common to biological molecules contribute to the pattern. The difference between the peaks is 0.5 m/z. The inverse is 2+, which is the charge state. The single addition of ^{13}C is the most abundant peak due to the large size of the protein. The probability of the presence of 1 ^{13}C is greater in a molecule of high molecular mass than in a small molecule, where the ^{12}C ion would be most abundant. The isotopic pattern shown here for peak A of Figure 4-8 is the same for peaks B and C.

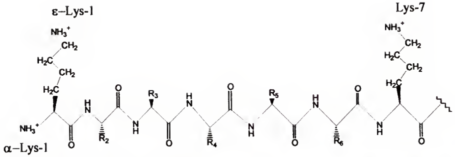


Figure 4-10. N-terminus end of the unmodified S-peptide illustrating the sites where modification can occur, α-Lys-1, ε-Lys-1 and ε-Lys-7.

If the close proximity of the modification to the peptide bond inhibits CID fragmentation, then modification at α-lys-1 would result in a slightly different CID fragmentation pattern from that of modification at ε-lys-1. Fragment ion y_{19}^{2+}, m/z 1020- $[M+2H]^{2+}$, corresponds to loss of the lys-1 if the modification occurred at lys-1. The y_{19}^{2+} ion was observed in the CID fragmentation pattern of the HPLC/ESI-mass chromatogram elution peak A but not in peak B or C.

The formation of the y_{19}^{2+} ion from peak A and not from peak B may indicate that peak A contains S-peptide modified at ε-lys-1 and peak B contains S-peptide modified at α-lys-1. Modification at ε-lys-1 is far enough away from the peptide backbone to allow lys-1 to be lost. Modification at α-lys-1 is closer to the peptide backbone, preventing loss of lys-1, and thus, ion m/z 1020- $[M+2H]^{2+}$ is absent from CID fragmentation of HPLC peak B.

To corroborate this theory, the modification found at lys-7 in peak C did not inhibit CID fragmentation at the adjacent peptide bond, as seen by the presence of the y_{13}^{1+}in peak C. The experimental data demonstrate the information that may be obtained from careful examination of the CID fragmentation patterns for each S-peptide ion. However,

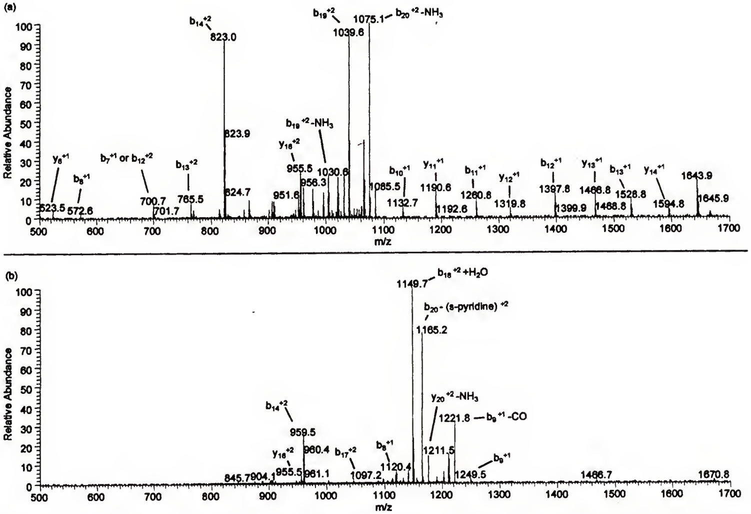


Figure 4-11. CID fragmentation spectra of a) unmodified S-peptide, m/z 1083-[M + 2H]$^{2+}$ and b) singly SMPT-modified S-peptide, m/z 1220-[M + 2H]$^{2+}$, peak A from Figure 4-8. Ion assignments are labeled according to conventional nomenclature. A complete listing of each assigned ion for unmodified S-peptide and singly SMPT-modified S-peptide for peaks A, B and C from Figure 4-8 is shown in Tables 4-3 through 4-6.

Table 4-3: Unmodified S-peptide fragment ions, MS^n of m/z 1083.50-$[M+2H]^{2+}$

m/z	b-ions[a]	m/z	y ions[b]
572.6	b_6^{+1}	434.5	y_5^{+1}
700.7	b_7^{+1} or b_{12}^{+2}	523.5	y_6^{+1}
765.6	b_{13}^{+2}	824.7	y_{15}^{+2}
823.1	b_{14}^{+2}	906.7	y_9^{+1}
866.4	b_{15}^{+2}	955.5	y_{18}^{+2}
910.1	b_{16}^{+2}	1019.5	y_{19}^{+2}
951.6	b_{17}^{+2}-NH_3	1190.6	y_{11}^{+1}
961.4	b_{17}^{+2}	1319.8	y_{12}^{+1}
976.8	b_9^{+1}	1466.8	y_{13}^{+1}
995.1	b_{18}^{+2}-NH_3	1594.8	y_{14}^{+1}
1004.1	b_{18}^{+2}	1666.1	y_{15}^{+1}
1030.6	b_{19}^{+2}-NH_3	1737.1	y_{16}^{+1}
1039.6	b_{19}^{+2}	1909.3	y_{18}^{+1}
1075.1	b_{20}^{+2}-NH_3		
1132.8	b_{10}^{+1}		
1260.8	b_{11}^{+1}		
1397.8	b_{12}^{+1}		
1528.9	b_{13}^{+1}		
1643.9	b_{14}^{+1}		

[a] "b-ions" are fragment ions generated from cleavage at the amide bonds with the charge retained on the N-terminal end of the peptide. The subscript indicates the amide bond where fragmentation occurred, counted from the C-terminal.

[b] "y-ions" are fragment ions generated from cleavage at the amide bonds with the charge retained on the C-terminal end of the peptide. The subscript indicates the amide bond where fragmentation occurred, counted from the N-terminal.

Table 4-4: Singly Modified S-peptide, Peak A, MS^n of m/z 1220.00-$[M+2H]^{2+}$ [a]

m/z	b-ions[b]	m/z	y-ions[c]
703.4	**b_4^{+1} or b_{10}^{+2}**	**823.3**	**y_{15}^{+2}**
973.7	**b_7^{+1}**	**904.3**	**y_{17}^{+2}**
901.4	b_{13}^{+2}	906.7	y_9^{+1}
959.5	b_{14}^{+2}	**955.5**	**y_{18}^{+2}**
1002.9	b_{15}^{+2}	**1019.5**	**y_{19}^{+2}**
1045.9	b_{16}^{+2}	1190.6	y_{11}^{+1}
1097.4	b_{17}^{+2}	1319.7	y_{12}^{+1}
1120.4	b_8^{+1}	1466.8	y_{13}^{+1}
1141.3	b_{18}^{+2}	**1594.8**	**y_{14}^{+1}**
1149.8	b_{18}^{+2}+H_2O	**1665.8**	**y_{15}^{+1}**
1165.1	b_{20}^{+2}-(s-pyr)	**1720.1**	**y_{16}^{+1}-NH_3**
1176.1	b_{19}^{+2}	**1736.9**	**y_{16}^{+1}**
1211.5	b_{20}^{+2}-NH_3	**1909.9**	**y_{18}^{+1}**
1221.5	b_9^{+1}-CO		
1249.5	b_9^{+1}		
1406.5	b_{10}^{+1}		
1533.8	b_{11}^{+1}		
1670.8	b_{12}^{+1}		
1801.7	b_{13}^{+1}		
1917.8	b_{14}^{+1}		

[a] Bolded ions indicate those which pinpoint site of modification. In this case ions indicate modification at lysine 1.

[b, c] See definition in Table 4-3.

Table 4-5: Singly Modified S-peptide, Peak B, MS^n of m/z 1220.00-[M+2H]$^{2+}$ [a]

m/z	b-ions[b]	m/z	y-ions[c]
513.6	**b_2^{+1}-NH$_3$**	523.5	y_6^{+1}
531.6	**b_2^{+1}**	638.4	y_7^{+1}
549.4	**b_2^{+1}+H$_2$O**	751.8	y_8^{+1}-NH$_3$
616.7	**b_3^{+1}-NH$_3$**	769.5	y_8^{+1}
631.9	**b_3^{+1}**	**904.3**	**y_{17}^{+2}**
703.4	**b_4^{+1} or b_{10}^{+2}**	906.6	y_9^{+1}
722	**b_4^{+1}+H$_2$O**	**947**	**y_{18}^{+2}-NH$_3$**
774.6	**b_5^{+1}**	**955.5**	**y_{18}^{+2}**
845.7	**b_6^{+1}**	1034.9	y_{10}^{+1}
902.1	b_{13}^{+2}	1211.5	y_{20}^{+2}-NH$_3$
959.5	b_{14}^{+2}	1303.6	y_{12}^{+1}-NH$_3$
1003.2	b_{15}^{+2}	1319.7	y_{12}^{+1}
1046.7	b_{16}^{+2}	1466.8	y_{13}^{+1}
1088.2	b_{17}^{+2}-NH$_3$	**1594.8**	**y_{14}^{+1}**
1097.2	b_{17}^{+2}	**1665.8**	**y_{15}^{+1}**
1120.4	b_8^{+1}	**1736.9**	**y_{16}^{+1}**
1141.3	b_{18}^{+2}	**1909.9**	**y_{18}^{+1}**
1149.8	b_{18}^{+2}+H$_2$O		
1165.1	b_{20}^{+2}-(s-pyr)		
1176.1	b_{19}^{+2}		
1211.5	b_{20}^{+2}-NH$_3$		
1221.5	b_9^{+1}-CO		
1249.5	b_9^{+1}		
1405.7	b_{10}^{+1}		
1533.8	b_{11}^{+1}		
1670.8	b_{12}^{+1}		
1801.7	b_{13}^{+1}		
1917.8	b_{14}^{+1}		

[a] Bolded ions indicate those which pinpoint site of modification. In this case, ions indicate modification at lysine 1.

[b, c] See definition in Table 4-3.

Table 4-6: Singly Modified S-peptide, Peak C, MS^n of m/z 1220.00-[M+2H]$^{2+}$ [a]

m/z	b-ions[b]	m/z	y-ions[c]
572.4	**b_6^{+1}**	523.5	y_6^{+1}
902.1	b_{13}^{+2}	638.4	y_7^{+1}
959.5	b_{14}^{+2}	751.8	y_8^{+1}-NH$_3$
1003.2	b_{15}^{+2}	769.5	y_8^{+1}
1046.7	b_{16}^{+2}	888.5	y_9^{+1}-NH$_3$
1097.2	b_{17}^{+2}	906.7	y_9^{+1}
1120.4	b_8^{+1}	**1004.1**	**y_{16}^{+2}**
1140.8	b_{18}^{+2}	**1091.4**	**y_{19}^{+2}**
1149.8	b_{18}^{+2}+H$_2$O	1211.5	y_{20}^{+2}-NH$_3$
1165.1	b_{20}^{+2}-(s-pyr)	1319.8	y_{12}^{+1}
1176	b_{19}^{+2}	1467.8	y_{13}^{+1}
1211.5	b_{20}^{+2}-NH$_3$	**1868.7**	**y_{14}^{+1}**
1221.5	b_9^{+1}-CO	**1938.8**	**y_{15}^{+1}**
1249.5	b_9^{+1}		
1405.9	b_{10}^{+1}		
1533.8	b_{11}^{+1}		
1670.8	b_{12}^{+1}		
1802.9	b_{13}^{+1}		
1916.8	b_{14}^{+1}		

[a] Bolded ions indicate those which pinpoint site of modification. In this case, ions indicate modification at lysine 7.

[b, c] See definition in Table 4-3.

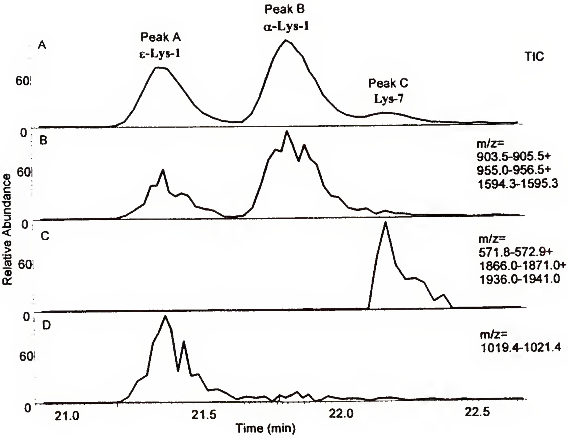


Figure 4-12. HPLC/ESI-mass chromatograms. a) Base peak for all m/z 1220-[M + 2H]$^{2+}$ ions, as in Figure 4-8. b) Mass selection of some ions that correspond to modification at Lys-1 only. The elution times of these ions indicate they are only present in the first 2 base peaks, A and B. c) Mass selection of some ions that correspond to modification at Lys-7 only. The elution times of these ions indicate they are only present in the last base peak, C. d) Mass selection of ion y_{19}^{2+} possibly corresponding to modification at ε-Lys-1. The elution time of this ion indicates it is only present in base peak A.

Edman sequencing of separated S-peptides is required for the conclusive assignment of modification at either the ε- or α-amino on lys-1.

Subtilisin Digest of Singly SMPT-Modified RNase A

In order to increase the amount of RNase A modified at a single site, the modified RNase A solution was separated by offline HPLC following Bio-Rex 70 chromatography. HPLC fractions that contained only singly modified RNase A (peaks A and B) were collected and digested by subtilisin (see Figure 2-21 and Figure 2-22). HPLC/ESI/QIT mass selection of ions corresponding to unmodified and modified S-peptide and unmodified and modified S-protein were plotted with respect to elution time. Offline HPLC peak B digestion resulted in the presence of singly modified S-protein with unmodified S-peptide (Figure 4-13). This result was expected as tryptic digestion in Chapter 3 showed a high propensity for modification at Lys-37. Offline HPLC of the peak A digest resulted in the presence of unmodified S-protein. It was expected that the modified S-peptide would be present because the sample was shown to be singly modified prior to digestion. However, peak A showed no singly modified S-peptide, m/z 1220-$[M + 2H]^{2+}$, as well as no unmodified S-peptide, m/z 1083-$[M + 2H]^{2+}$ (Figure 4-14). The absence of the modified S-peptide ion (m/z 1220-$[M + 2H]^{2+}$) indicated that either the modification had been cleaved from the S-peptide or the modified S-peptide had been chemically changed to an ion with an unidentified mass.

The data from the subtilisin digest does not directly parallel the selectivity attributed to lysine residues from the tryptic digest in Chapter 3, again indicating the possibility of cleavage and/or chemical alteration in the electrospray source or during the subtilisin digest.

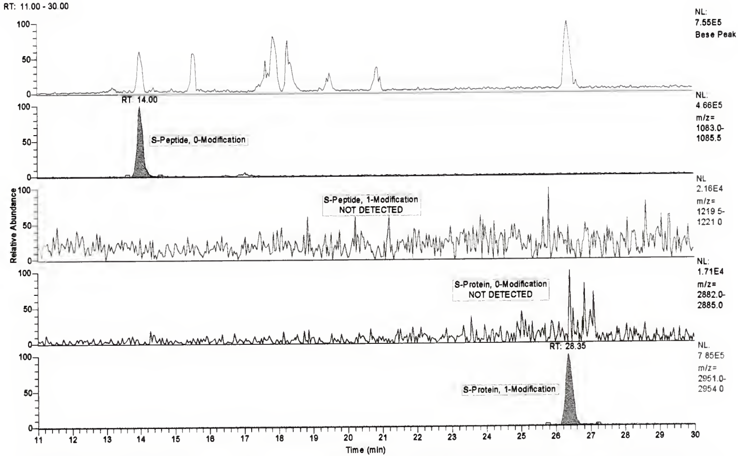


Figure 4-13. HPLC/ESI-mass chromatograms of SMPT-modified RNase A that had been previously separated by offline HPLC to remove any unmodified or multiply modified RNase A. The sample digested is from Figure 2-21, peak B. Mass selections correspond to unmodified and singly modified S-peptides and S-proteins and indicate modification on the S-protein only.

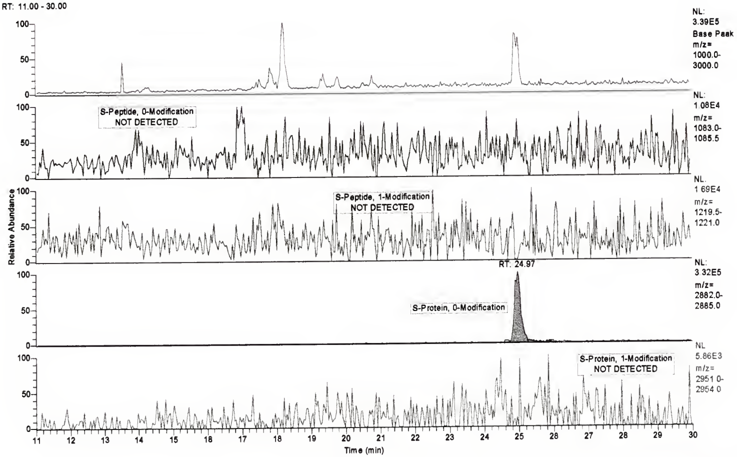


Figure 4-14. HPLC/ESI-mass chromatograms of SMPT-modified RNase A that had been previously separated by offline HPLC to remove any unmodified or multiply modified RNase A. The sample digested is from Figure 2-21, peak A. Mass selections correspond to unmodified and singly modified S-peptides and S-proteins and indicate no modification to the S-protein, and also no modification to the S-peptide. The modification may be cleaved or chemically altered during digestion with subtilisin such that mass selection for singly modified S-peptide shows a negative result. The top elution chromatogram is the base elution of all ions and shows the presence of other ions. These may be chemically altered singly modified S-peptide.

Conclusion

Cleavage of RNase A by subtilisin-Carlsberg has been quantified by HPLC/ESI-MS and has been shown to cleave between residues 20-21 and residues 21-22 as previously reported in the literature, but also between residues 16-17, 18-19 and 19-20. Also demonstrated is the effect of chemical modification to the RNase A substrate at lysines 1 and 7 on the specificity of subtilisin. Digestion of RNase A with subtilisin was shown to result in cleavage at various peptide bonds along the RNase A backbone with inconsistent selectivity for each peptide bond in trials with the same reaction conditions. Inconsistent selectivity was also found for SMPT-modified S-peptide. Such a result may enable a higher degree of understanding in experiments that exploit the S-peptide/S-protein noncovalent attraction and activity.

Digestion of the modified and unmodified RNase A by subtilisin followed by examination by HPLC/ESI/QIT-MS and MS^n enabled further investigation of modification at lysines 1 and 7. MS^n on singly modified S-peptide indicated modification at the ε- and α-amine positions on lysine 1.

SMPT-modification at Lys-1 was not predominant in the subtilisin digest results as it was in the tryptic digest. These inconsistent results may be due to a chemical reaction of the modified S-peptide during subtilisin digestion or in the electrospray source, such that significant amounts of modified S-peptide at the expected m/z are not seen. Despite the problems encountered in mass spectrometric mapping of the modifications on RNase A, it remains a method complementary to traditional sequencing.

CHAPTER 5
BIOCONJUGATION OF RNASE A TO A SYNTHETIC COFACTOR: PRELIMINARY WORK

Introduction

In this chapter, preliminary work is presented on two different strategies for the completion of RNase A bioconjugated to a synthetic cofactor. The first strategy involves the use of singly SMPT-modified RNase A prepared in chapter 2. Despite the lack of unequivocal knowledge of the site of modification demonstrated in chapters 3 and 4, linkage of the single SMPT modification on RNase A to a reagent of interest is presented. The reagent of interest is a copper dipyridylamine cofactor (Figure 5-1).

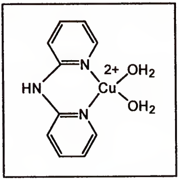


Figure 5-1. $[(2,2'\text{-dipyridylamine})Cu(OH_2)_2]^{2+}$, metal cofactor to be bioconjugated to RNase A.

Cisdiaquo-metal complexes have shown efficient catalysis for hydrolysis of activated and unactivated esters such as p-nitrophenyl acetate (175) and methyl acetate (176), respectively. The proposed mechanism involves coordination of the substrate to the metal followed by intramolecular metal hydroxide attack on the metal-bound ester forming a four-membered ring intermediate (Figure 5-2). This bifunctional activation allows for catalysis in hydrolysis of not only unactivated esters, but also for nitriles, amides, and phospho- mono, di- and triesters (176). The complex [(2,2'-dipyridylamine)Cu$(OH_2)_2]^{2+}$ (Cu(DPA)) (Figure 5-1) has been chosen as the metal cofactor complex to be linked to RNase A. The complex binds Cu^{2+} more tightly (K = 1.15 x 10^8 mol^{-1} L) than it binds H^+ (K = 1.38 x 10^7 mol^{-1} L) (177). Therefore, according to Chin *et al.* (178), Cu^{2+} does not dissociate from the 2,2'-dipyridylamine over a wide pH range. Chin *et al.* (176) have reported 10^5 – 10^6 rate enhancement by the complex for ester hydrolysis. Thus, its covalent attachment to RNase A would create a semi-synthetic enzyme with potentially novel catalytic properties such as deoxyribonuclease activity in a system that is normally inhibited by DNA.

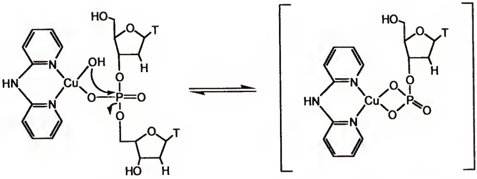


Figure 5-2. Substrate coordinated to metal complex, [(2,2'-dipyridylamine)Cu(O_2H)], followed by possible four-coordinate intermediate.

In order to incorporate the Cu(DPA) into RNase A, 2,2'-dipyridylamine (DPA) is first functionalized to prepare *N*,*N*-dipyridyl-4-amino-1-butanethiol (DPA thiol) reagent without copper II (see Figure 5-3). The DPA thiol is then exchanged with the SMPT modification on RNase A at the SMPT disulfide to covalently link the DPA to RNase A. A reaction scheme for the exchange is shown in Figure 5-3. Once the DPA has been linked to RNase A, the complex is metallated with copper II for completion of the bioconjugate. Preliminary work toward the goal of the final assembly of the bioconjugate is presented with incorporation of DPA into the singly modified SMPT-RNase A and its characterization by MALDI/TOF-MS and ESI/FTICR-MS.

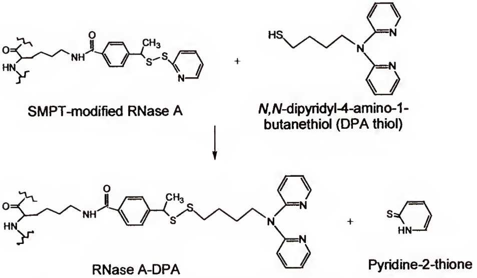


Figure 5-3. Reaction scheme for the exchange of the SMPT disulfide for the *N,N*-dipyridyl-4-amino-1-butanethiol (DPA thiol). Pyridine-2-thione is released.

The second strategy employed toward the goal of a bioconjugated RNase A involves the use of recombinant techniques. One goal of the research described in past chapters was to demonstrate the use of HPLC and mass spectrometry to guide and track chemical modification of a protein for the development of a system that could be bioconjugated to create a semi-synthetic enzyme. In general, development of successful bioconjugation systems have been accomplished through chemical modification of a uniquely modifiable residue occurring naturally in a protein or by the introduction of such a residue into a protein by site-directed mutagenesis (179-189).

Chapters 2 through 4 described the isolation and purification of chemically modified RNase A. HPLC and mass spectrometry were used to guide the process of development of this system, which is to be used as a base for bioconjugation. Problems outlined in the above chapters included modification at various sites on the protein that resulted in fractions of derivatized RNase A that appeared to be inseparable by standard chromatographic methods as well as complexities with ESI/FTICR-MS determination of site of modification. Such problems could be solved by choice of a different base protein with fewer sites where chemical modification may occur along with characterization by traditional sequencing techniques to complement analysis by mass spectrometry.

Development of a base protein to function as a semi-synthetic enzyme can also be accomplished through site-directed mutagenesis. This approach has been used by other researchers to introduce a residue that could be modified exclusively, eliminating the possibility of protein modification at different individual sites and with multiple reagent groups on a given protein molecule (179-189). As a complement to the chemical modification and characterization of RNase A presented above, site-directed mutagenesis has been used to introduce a unique residue, which can be later ligated to a functional group of interest. This technique circumvents the problem of multiple modifications to RNase A with chemical modification techniques and still establishes it as a base protein system for bioconjugation.

Site-directed mutagenesis is a technique used to engineer proteins. Protein synthesis at the amino acid level is, as yet, generally unsuccessful due to the lack of understanding of the compilation of forces that result in the folding of a protein into its native conformation. One approach to this problem has been to copy Nature and work at the level of DNA. Mutation of the gene that encodes for a protein of interest results in the expression of the mutation translated into the amino acid sequence and the substitution of the target residue or residues. This technique of synthesizing the DNA that contains the mutant that directs the amino acid mutation was first presented by Schott and Kossel in 1973 (190). Mutagenesis at a specific position in a DNA sequence was reported in a landmark paper by Hutchison *et al.* (191). Site-directed mutagenesis theory and techniques have been the subject of many reviews including those by Shortle (192,193), Smith (194), Fersht (195,196), Knowles (197) and Hutchison *et al.* (198).

The gene that encodes for RNase A was mutated at the codon for the target amino acid, phenylalanine-8, into the codon for the desired mutant amino acid residue, cysteine-8 (commonly expressed as F8C). Phenylalanine-8 was selected for mutation because of its proximity to the active site. Covalent linkage of DPA to a residue at position 8 would place the synthetic cofactor near the active site of the protein. Successful positioning of the DPA may result in its participation in catalytic activity once metallated with Cu^{2+}. It was anticipated that mutation of phe-8 to cys-8 would be starting point from which to pursue different mutations based on the ability of F8C RNase A to fold, to couple to the DPA and to bind substrates. The reaction scheme for linkage of DPA with the reagent *N, N*-dipyridyl-4-amino-1-butyl 2'-disulfide (DPA disulfide) to F8C RNase A is shown in Figure 5-4.

The characterization techniques of MALDI/TOF-MS were used for preliminary characterization of the recombinant RNase A. Problems commonly associated with the use of site-directed mutagenesis as a method for introduction of a uniquely modifiable residue for bioconjugation include protein folding into the correct conformation for activity and low yield of the mutant. Poor folding of the recombinant protein makes further bioconjugation pointless, as the semi-synthetic enzyme usually functions through exploitation of the natural binding capabilities of the base protein. Low yield of the recombinant protein severely reduces the amount of semi-synthetic enzyme available for characterization by crucial kinetic analyses with various substrates and for later evaluation as a potential therapeutic agent.

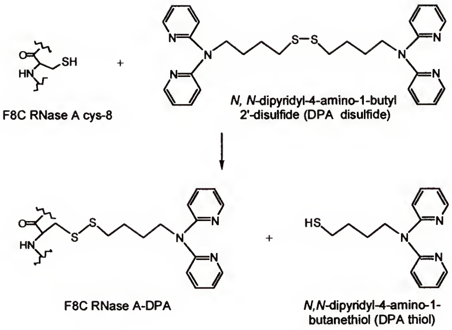

SH
F8C RNase A cys-8
N, N-dipyridyl-4-amino-1-butyl 2'-disulfide (DPA disulfide)
F8C RNase A-DPA
N,N-dipyridyl-4-amino-1-butanethiol (DPA thiol)

Figure 5-4. Reaction scheme for the coupling of cys-8 on F8C RNase A with *N, N*-dipyridyl-4-amino-1-butyl 2'-disulfide (DPA disulfide). *N,N*-dipyridyl-4-amino-1-butanethiol (DPA thiol) is released.

Nevertheless, the utility of site-directed mutagenesis for the introduction of a uniquely modifiable residue is an important adjunct to the chemical modification route used in previous chapters for the development of a base protein system for bioconjugation. Therefore, preliminary work on the mutation of RNase A, F8C, is described with characterization of the recombinant protein by MALDI/TOF-MS.

Experimental Procedures

Coupling of Singly SMPT-Modified RNase A and DPA Thiol

Singly SMPT-modified RNase A that was prepared in chapter 2 was coupled to *N*, *N*-dipyridyl-4-amino-1-butanethiol (DPA thiol), which had been synthesized previously by Celeste Regino. The SMPT-modified RNase A was dissolved in 50 mM EPPS buffer, pH 8.0 (Sigma). The concentration of SMPT-modified RNase A was approximated to be 1×10^{-5} M in 500 μL. To this solution was added the DPA thiol (1×10^{-6} M, 500 μL in methanol) drop-wise over the course of an hour under nitrogen. The solution was allowed to incubate for 6 hours at room temperature with stirring. The solution was then frozen at -80°C and stored prior to analysis by MALDI/TOF-MS and HPLC/ESI/QIT-MS.

Summary of PCR Based Mutagensis of Ribonuclease A F8C

The complete experimental procedure for the mutagenesis and isolation of RNase A F8C was performed by Lee Raley and is described in detail in the Appendix. A summary of these procedures is presented here.

The mutant ribonuclease was derived from the wild type made originally by Monika Huagg (199-201) in a pET 23 B+ plasmid. The mutant was created using a 5' mutagenic primer (IDT Corporation, Coralville, IA.), creating the F8C. The polymerase chain reaction (PCR) product was placed into a TOPO PCR 2.1 vector via the TOPO-TA kit (Invitrogen, Carlsbad, CA). The plasmid was transformed into a TOPO10 cell and plated. Colonies were selected by PCR. Colonies containing the correct plasmid were grown overnight. The plasmid was harvested using a Wizard Kit (Promega, Madison, WI). The plasmid was digested with restriction enzymes Nde I and EcoRI (New England Biolabs, Beverly, MA). The RNase A F8C insert was isolated by gel purification. The insert was then ligated into a Nde I and EcoRI digested pET 23 B+ vector (Novagen, Madison, WI). The vector was transformed into BL21(DE3)pLysS, *Escherichia coli* cells. The cells were plated on Luria-Bertani agar with ampicillin (LB amp) (Fisher, Springfield, NJ). The colonies were selected by PCR. Cells were grown in 10 mL of LB amp broth. Two milliliters of the 10 mL culture were used to inoculate 800 mL of Luria-Bertani* (LB*) media (Fisher). The rest was used to make glycerol cultures. The culture was grown at 37°C until the optical density at 600 nm (A600) was 0.6, followed by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO). The culture continued to grow for four hours. The cells were harvested by centrifugation and lysed by french press. The inclusion bodies were collected by centrifugation. They were then denatured and purified by carboxymethyl cationic exchange chromatography. The protein was refolded in glutathione buffer (Sigma) and concentrated. The protein was then placed on a uridine 2', 5'-and 3', 5'-diphosphate-agarose (pUp-agarose) (Sigma) column and purified. The RNase fractions were pooled, concentrated and lyophilized followed by storage at -6°C.

Digestion of F8C RNase A

F8C RNase A was digested with subtilisin-Carlsberg as described for native and modified RNase A in chapter 4. F8C RNase A was dialyzed against ammonium bicarbonate, 0.1 M, pH 8.0 prior to digestion to remove tris salts.

Coupling of F8C RNase A and DPA Disulfide

F8C RNase A was coupled to *N, N*-dipyridyl-4-amino-1-butyl 2'-disulfide (DPA disulfide), which had been synthesized previously by Celeste Regino. F8C RNase A was dissolved in 50 mM EPPS buffer, pH 8.0 (Sigma). The high concentration of tris salts in the lyophilized sample were removed by Amicon filtration (MW cutoff = 1000) against EPPS buffer. The concentration of F8C RNase A was approximated to be 1 x 10^{-5} M in 800 μL. This solution was added drop-wise over the course of an hour to the ligand, DPA disulfide, which was in excess and dissolved in 200 μL acetonitrile under nitrogen. The solution was allowed to incubate for 24 hours at room temperature with stirring. Subsequently, the acetonitrile was removed by evaporation under nitrogen and methanol was added until all precipitate dissolved. The solution was then frozen at -6°C and stored prior to analysis by HPLC/ESI/QIT-MS.

MALDI/TOF-MS

MALDI/TOF-MS data were collected by David H. Powell. MALDI/TOF-MS data were acquired by using a PerSeptive Voyager DE-Pro Mass Spectrometer (PE

Biosystems, Framingham, MA) with a 337 nm nitrogen laser run in a linear mode. The matrix used was 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid) (Sigma, St. Louis, MO). The sinapinic acid matrix was prepared in acetone containing 0.1% TFA. Two µL of matrix was applied to the MALDI plate followed by 2 µL of sample. The samples were either spotted directly on the dried matrix (when in 50% methanol / 50% H_2O with 0.1% TFA added) or spotted after desalting by chromatography using ZipTips™, C-18, (Millipore, Bedford, MA). A ZipTip™ was used to desalt samples by first wetting the tip resin with 5 µL of acetonitrile followed by rinsing two times with 5 µL each of 0.1% TFA in H_2O. Two µL of sample was then loaded onto the ZipTip™ followed by rinsing with 2 µL of 0.1% TFA in H_2O. The sample was then eluted onto the MALDI plate on top of the dried matrix with 2 µL of 50% acetonitrile / 50 % H_2O with 0.1% TFA added.

HPLC/ESI/QIT-MS

HPLC/ESI/QIT-MS data were collected by Jodie V. Johnson. HPLC/ESI-MS and data were collected as described in chapter 4 with changes detailed here. A binary HPLC mobile phase system was used: buffer A consisted of 1% acetic acid (Fisher) and 20 mM ammonium acetate (Sigma) in H_2O, while buffer B was 0.5% acetic acid in 49.75% methanol and 49.75% isopropanol (all Fisher). Chromatographic separation was achieved with a Waters (Milford, MA) Symmetry Shield 3µ, RP-18 column (2.1 mm i.d. x 150 mm; with a guard column). The elution gradient was as follows: 0 min (A:B=95:5), 5 min (A:B=95:5), 20 min (A:B=70:30), 30 min (A:B=65:35), 40 min(A:B=5:95); 55 min (A:B=5:95); 65 min (A:B=95:5) ; 80 min (A:B=95:5). The gradient flow was 0.2 mL/min and the column effluent was not split prior to the ESI source.

Results and Discussion

Strategy 1: Coupling of SMPT-Modified RNase A to DPA Thiol

The incorporation of a synthetic metal cofactor into RNase A would create a bioconjugate with potentially novel catalytic properties, possibly enhancing the activity or altering it through the cleavage of new substrates. The cofactor [(2,2'-dipyridylamine)Cu$(OH_2)_2$]$^{2+}$ (Cu(DPA)) was selected to be covalently linked to RNase A. For this purpose, the non-metallated complex, 2,2'-dipyridylamine, was functionalized by Celeste Regino to form the butanethiol derivative, *N*, *N*-dipyridyl-4-amino-1-butanethiol (DPA thiol). The DPA thiol was then to be exchanged with the pyridine thiol ring on SMPT-modified RNase A. In order to form a well-characterized bioconjugate, the SMPT-modified RNase A to which the DPA thiol would be linked should be homogeneous in number and location of SMPT modification. Experimental results in chapters 2, 3 and 4 demonstrated that the high number of modifiable residues in RNase A along with the low selectivity of the SMPT reagent resulted heterogeneous modification. Despite multi-dimensional separations, experimental results were consistent with heterogeneous modification in fractions of RNase A that were demonstrated to contain only singly modified protein.

Despite the heterogeneity of the singly SMPT-modified RNase A, the ability to incorporate the 2,2'-dipyridylamine cofactor was pursued. Reaction conditions for the coupling were varied with respect to organic solvent to aqueous solvent ratios, choice of organic and aqueous solvents, buffer concentration, concentration of reactants, delivery system of the DPA thiol, and reaction time. The conditions described in the experimental section resulted in successful exchange of the pyridine thiol ring on the SMPT with the DPA thiol. MALDI/TOF-MS was used to demonstrate the mass change from singly SMPT-modified RNase A (Figure 5-5) to RNase A coupled to a single DPA through the SMPT/butyl linker (Figure 5-6).

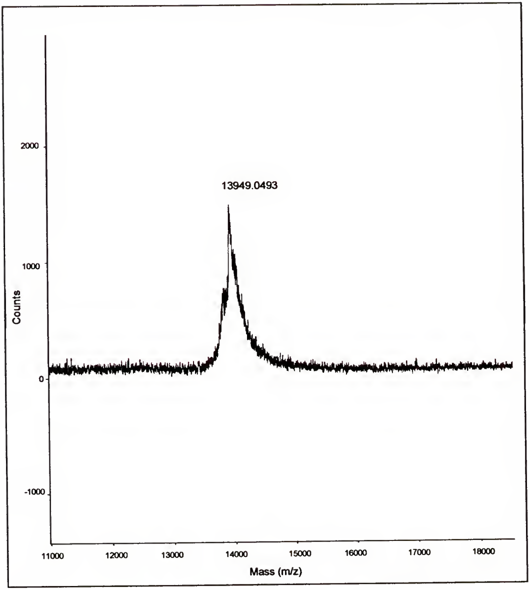


Figure 5-5. MALDI/TOF-MS of singly SMPT-modified RNase A. M/z 13949.05-$[M + H]^{1+}$ corresponds to the single addition of SMPT to RNase A. The sample was separated first on Bio-Rex 70 resin followed by HPLC.

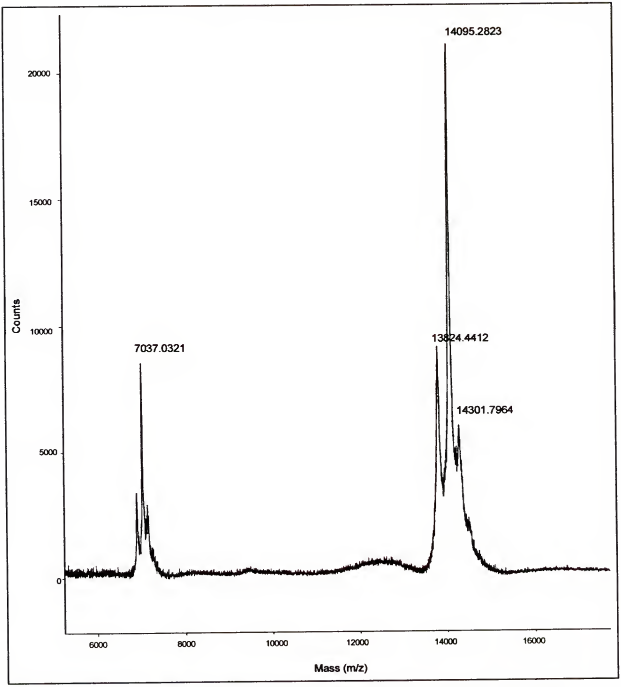


Figure 5-6. MALDI/TOF-MS of singly SMPT-modified RNase A coupled to DPA thiol. M/z 14095.28-$[M + H]^{1+}$ and m/z 7037.03-$[M + 2H]^{2+}$ correspond to the exchange of one DPA thiol for one pyridine-2-thione on the single SMPT coupling reagent linked to RNase A. The small peaks on either side of the major peak are unidentified, yet RNase A related.

The expected m/z for a single SMPT modification was 13954.34-$[M + H]^{1+}$ in Figure 5-5. The measured value was found to be m/z 13949.05-$[M + H]^{1+}$. The mass accuracy for mass values in this range was approximately ± 0.1% or ± 13 Da. The mass accuracy was good enough to detect the changes in m/z for the modifications of interest. The MALDI/TOF-MS mass accuracy was offset by the high sensitivity to picomole/μL or lower quantities of protein.

In Figure 5-6, the coupling of the DPA thiol to the single SMPT-modified RNase A was demonstrated by the intense peak at m/z 14095.28-$[M + H]^{1+}$ and the corresponding peak at m/z 7037.03-$[M + 2H]^{2+}$. The expected value for a DPA thiol addition to singly SMPT-modified RNase A was m/z 14098.53-$[M + H]^{1+}$. These results show the incorporation of a single DPA complex into RNase A.

HPLC/ESI/QIT-MS was also used to observe the singly SMPT-modified RNase A and the fraction that had been coupled to the DPA. First, the singly SMPT-modified RNase A was examined. Multiply protonated ions were found as shown in Figure 5-7 with $[M + 7H]^{7+}$ being the most intense. No native or multiply SMPT-modified RNase A were detected during the HPLC elution. The mass change for one SMPT modification to native RNase A is 273.3 Da, corresponding to an m/z change of 39.0 at $[M + 7H]^{7+}$. Second, the singly SMPT-modified RNase A coupled to DPA was examined as shown in Figure 5-8. Again, multiply protonated ions were found with $[M + 8H]^{8+}$ as the most intense ion. The mass change for one SMPT coupled to DPA is 417.5 Da, corresponding to an m/z change of 52.2 at $[M + 8H]^{8+}$. These results demonstrate once again the incorporation of a single DPA complex into RNase A.

Strategy 2: Characterization of F8C RNase A by MALDI/TOF-MS

The second strategy for the assembly of a bioconjugated RNase A is the introduction of a uniquely modifiable residue by site-directed mutagenesis. Subsequent

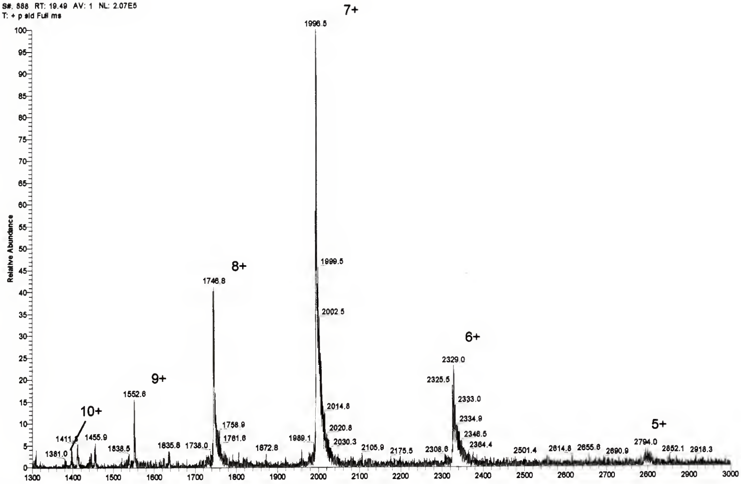


Figure 5-7. HPLC/ESI/QIT-MS of singly SMPT-modified RNase A at $[M+5H]^{5+}$, $[M+6H]^{6+}$, $[M+7H]^{7+}$, $[M+8H]^{8+}$, $[M+9H]^{9+}$and $[M+10H]^{10+}$ states. The expected value for $[M+8H]^{8+}$was m/z 1745.29 with a measured value of m/z 1746.8-$[M+8H]^{8+}$. The sample was separated first on Bio Rex 70 resin followed by HPLC offline.

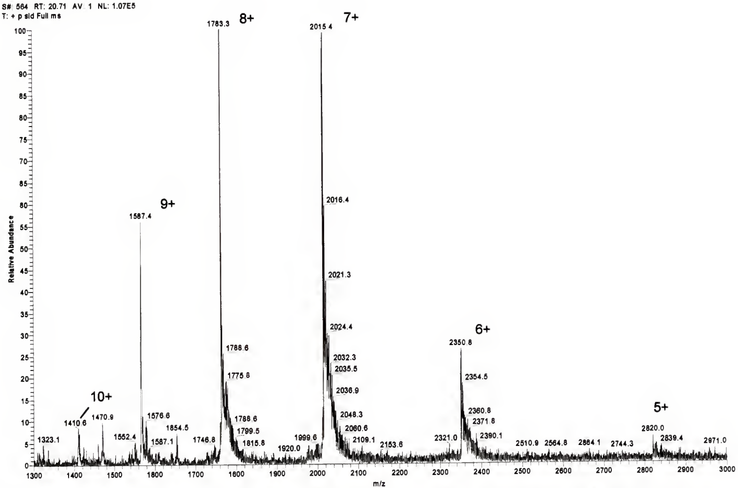


Figure 5-8. HPLC/ESI/QIT-MS of singly SMPT-modified RNase A linked to DPA at $[M+5H]^{5+}$, $[M+6H]^{6+}$, $[M+7H]^{7+}$, $[M+8H]^{8+}$, $[M+9H]^{9+}$and $[M+10H]^{10+}$ states. The expected value for $[M+8H]^{8+}$was m/z 1763.32 with a measured value of m/z 1763.3-$[M+8H]^{8+}$.

linkage of the mutated residue, in this case cysteine-8 to the synthetic cofactor, 2,2'-dipyridylamine through disulfide exchange would allow for the incorporation of the DPA into RNase A at a known location. Because of low yield of the F8C RNase A, the high sensitivity of MALDI/TOF-MS was needed for detection of the mutant. The isolation of the F8C RNase A was verified by the MALDI/TOF-mass spectrum shown in Figure 5-9. The expected m/z value for the F8C mutant was 13769.2-$[M + H]^{1+}$. The experimental m/z values were found to be 13769.7-$[M + H]^{1+}$ and 6888.1-$[M + 2H]^{2+}$. These m/z values reflect the loss of phenylalanine and gain of cysteine and methionine. Methionine is located at the N-terminus of the protein as a result of the expression of the protein in a prokaryotic cell line.

Digestion of F8C RNase A by subtilisin was performed as described in chapter 4 for native RNase A. Cleavage of F8C RNase A into the F8C-S-peptide and S-protein followed by HPLC/ESI/QIT-MS would allow for the separation of the two polypeptides with subsequent CID fragmentation of the F8C-S-peptide ion. Difficulty in HPLC/ESI/QIT-MS analysis of the digested F8C RNase A was the result of the exceptionally low concentration ($< 10^{-12}$ mol/μL) of F8C RNase A. Analysis of the digested F8C RNase A by MALDI/TOF-MS was attempted and was unsuccessful.

Linkage of the DPA to F8C RNase A was attempted as shown in Figure 5-4. Analysis by HPLC/ESI/QIT-MS and MALDI/TOF-MS did not determine whether the exchange was successful for the reason of low concentration noted above. Once again, a higher initial concentration of F8C RNase A would allow for the success of the coupling reaction to be determined.

Conclusion

Despite the heterogeneity of fractions containing singly SMPT-modified RNase A, the coupling of the modified protein to a synthetic cofactor, DPA was demonstrated. The

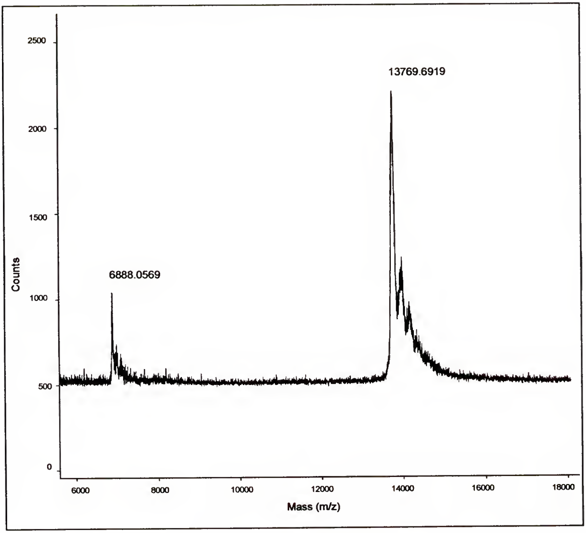


Figure 5-9. MALDI/TOF-MS of F8C RNase A. M/z13769.7-$[M + H]^{1+}$ and 6888.1-$[M+2H]^{2+}$ correspond to F8C RNase A. The expected value for $[M + H]^{1+}$ was m/z 13769.2. These m/z values reflect the loss of phenylalanine and gain of cysteine and methionine.

ability to incorporate the DPA illustrates the assembly of a bioconjugate. This example may be applied to other proteins with fewer sites of modification and/or with a modification reagent with increased specificity to create a semi-synthetic system that may possess novel catalytic activity.

The use of a recombinant protein such as F8C RNase A represents another possibility for the assembly of a bioconjugate. The isolation of F8C RNase A has been demonstrated. Despite this positive result obtained from MALDI/TOF-MS analysis, detection of neither digested nor coupled F8C RNase A was achieved. While higher concentrations of F8C RNase A to be digested would solve the detection problem seen with mass spectrometric analysis, low concentrations of recombinant protein is a limitation inherent to site-directed mutagenesis and that presents a challenge to the use of a recombinant protein as a base system for bioconjugation.

CHAPTER 6
GENERAL CONCLUSIONS

The design of functional enzymes has long been a goal of medical and biochemical research. The ability to synthesize an "artificial" enzyme to perform a reaction with the specificity and rate of a natural enzyme would introduce revolutionary advances in industrial and pharmaceutical product development. Because the complex factors that contribute to enzyme activity are not yet reproducible by synthetic means, the goal of an artificial enzyme remains elusive.

Research efforts toward this goal have incorporated many different strategies. One strategy has been the incorporation of a synthetic catalytic cofactor into a naturally occurring enzyme in an attempt to alter or enhance its activity, resulting in a "semi-synthetic enzyme." Introduction of such a cofactor has been generally approached by different methods including chemical modification. This method has been accomplished by modification at a unique residue in a given protein system that results in linkage of the cofactor to that residue exclusively. Because enzymes with a uniquely modifiable residue are few, introduction of such a residue by site-directed mutagenesis expands the number of base-protein systems that can be exploited.

Another method of chemical modification is the modification of a protein that does not possess a uniquely modifiable residue followed by separation of the reaction products to obtain fractions that contain protein that has been modified at a known site. The system can then be used as a bioconjugate, incorporating the cofactor of interest into the protein. This approach allows for the exploitation of many proteins as base systems for modification and theoretically presents the possibility of having many variations of the same base-enzyme system with the cofactor at different locations.

The research presented in this dissertation represents the method development for the preparation and characterization of an enzyme as potential bioconjugate system. The enzyme used for the method development was bovine pancreatic ribonuclease A (RNase A). Because RNase A is a well-characterized and readily available enzyme, it was suitable for the development of bioanalytical methods that may be applied to other chemically modified enzymes.

Chapter two focused on the selection of a reagent with which to modify RNase A. The first reagent chosen was Traut's reagent, 2-iminothiolane. Mass spectrometric analysis demonstrated extensive modification of RNase A by Traut's reagent under various reaction conditions. Covalent linkage of the Traut's reagent to an RNase A lysine residue resulted in the retention of positive charge at the modification site, making the necessary separations by available means difficult. For these reasons, NHS-acetate was used as the next reagent for RNase A modification.

NHS-acetate neutralizes lysine residues upon modification of RNase A, making separation of the derivatized product by ion-exchange chromatography possible. The acetate-modified RNase A was then used to develop a chromatographic separation procedure for obtaining fractions of RNase A modified with different numbers of acetate. Mass spectrometry was used to verify the extent of modification of the separated, derivatized protein, thereby demonstrating the possibility of isolating singly modified RNase A.

Reagents with further functionality, N-succinimidyl-3-[2-pyridyldithio]propionate (SPDP) and 4-succinimidyloxycarbonyl-methyl-ε-[2-pyrdyldithio]-toluene (SMPT) were then used to modify RNase A. Both reagents appeared to have similar reactivity with

RNase A lysine residues as did NHS-acetate. Difficulty in determination of mass changes upon modification with SPDP by mass spectrometric analysis made SMPT the final reagent of choice for developing the base-protein system for future bioconjugation.

The chromatographic procedures developed for the acetate-modified RNase A were used to separate the SMPT-modified RNase A. Mass spectrometric analysis demonstrated that the highest abundance of modified RNase A was in the form of single SMPT additions. Analysis of chromatographically separated SMPT-modified RNase A showed the isolation of fractions that contained predominantly singly modified RNase A, but suggested modification had occurred at different locations. A second dimension of separation was added by the use of HPLC (offline). Mass spectrometric analysis clearly showed isolation of singly SMPT-modified RNase A. Examination of the location of modification was the subject of the next two chapters.

The focus of chapter three was the mapping of SMPT modifications to RNase A by using tryptic digestion and mass spectrometric analysis of the partial peptide sequence digest products. Digestion was performed first on the unseparated SMPT-RNase A digestion products along with a native control to examine the selectivity of the different lysine residues for the SMPT reagent under the reaction conditions used. Analysis of native and modified RNase A tryptic digests by ESI/FTICR-MS resulted in the identification of the sites of modification. Semi-quantitative results of the reactivity of certain lysine residues toward the coupling reagent SMPT were possible. Two sites (lysines 1 and 37) were highly reactive, while three sites (lysines 41, 61 and 104) appeared to be unreactive under the conditions used.

Difficulty in using ESI/FTICR-MS as the sole means of determining relative abundances of modified and unmodified peptide segments became apparent, including the possible neutralization of certain peptide segments in the electrospray source either in the solution/droplet phase or the gas phase. The problems that arose in relating

ESI/FTICR-MS relative ion abundance in the gas phase to relative concentration in solution became apparent with data analysis. The issues discussed in chapter three convey the caution that must be taken when attempting to make such a relation. This statement was supported by the second digestion that was performed on SMPT-modified RNase A that had been further separated by offline HPLC. Before digestion, the sample was shown to contain only singly modified RNase A by ESI/FTICR-MS. Following digestion, however, experimental results were consistent with heterogeneity with respect to modification site.

However, the analytical technique did result in the collection of semi-quantitative data and allowed for characterization of the chemical modification of RNase A with SMPT. Demonstrated by this method was that predominant modification occurred at two sites, lysines 1 and 37. Due to their close proximity to each other, modification at these sites would make separation by ion-exchange chromatography difficult, and this was not achieved in the work presented above.

Further mapping of SMPT modified RNase A was attempted by proteolytic digestion with subtilisin followed by HPLC/ESI/QIT-MS analysis, and this was the subject of chapter four. Before digestion of SMPT-modified RNase A was attempted, the specificity of cleavage by subtilisin for RNase A peptide bonds was evaluated. It was demonstrated by HPLC/ESI/QIT-MS analysis that significant cleavage (up to 14% ± 3%) occurred at peptide bonds other than the main cleavage site between residues 20 and 21. This cleavage at various amide bonds was found with SMPT-modified RNase A as well.

Examination of the SMPT-modified RNase A by digestion with subtilisin demonstrated again that modification occurred at various locations on the protein. Because the results of the tryptic digest had shown that significant modification had occurred at lysine 1, one of the subtilisin digestion products, modified S-peptide (residues 1-20), was evaluated by HPLC/ESI/QIT-MS^n. The ion that corresponded to a single SMPT modification to the S-peptide was m/z 1220 $[M + 2H]^{2+}$. This ion was shown to elute at three different retention times on reverse-phase HPLC. Each ion corresponding to the different retention times was fragmented separately by CID as well as the unmodified S-peptide. Subsequent analysis of their fragmentation spectra revealed that each ion at a given retention time possessed SMPT modification at a different site, either α-lysine 1, ε-lysine 1 or ε-lysine 7. Such an analysis demonstrated the capability of mass spectrometry to provide in depth sequencing information on a target peptide.

While chapters three and four examined the reaction product of the SMPT modification of RNase A, chapter five focused on further assembly of the bioconjugate. Despite the lack of isolation of RNase A that had been modified by SMPT at a specific lysine residue, it was useful to demonstrate the incorporation of a synthetic cofactor into the RNase A. Fractions of RNase A that were shown to contain singly SMPT-modified RNase A, while heterogeneous with respect to site of modification, were linked to the synthetic cofactor, DPA thiol. The thiol exchange of the DPA thiol reagent with pyridine-2-thione resulted in the covalent attachment of DPA to RNase A through the SMPT/butyl linkage, which was demonstrated by MALDI/TOF-MS and HPLC/ESI/QIT-MS. The success of this reaction illustrates the possibility of the incorporation of a cofactor into a protein through a chemically modified residue. The next step in the bioconjugate development will be the addition of Cu^{2+} to the DPA cofactor. Evaluation of the metallated semi-synthetic system will be accomplished through kinetic analyses with various substrates.

As a complement to the chemical modification of RNase A, preliminary work was begun on the characterization of RNase A that had been mutated by site-directed mutagenesis. This approach to the introduction of a uniquely modifiable residue was also a focus of chapter five. The success of the isolation of a recombinant RNase A, F8C, was demonstrated by MALDI/TOF-MS. Further characterization of F8C RNase A was pursued in this chapter but was unsuccessful due to low concentrations of the recombinant protein. The same problem prevented analysis of the F8C RNase and DPA disulfide reaction products. However, this problem can be overcome with increased concentrations of F8C RNase A. Another possibility is mutation of RNase A at a residue other than phe-8. Such a mutation may give the recombinant protein more stability, maintain native conformation and/or place the synthetic cofactor in a more advantageous location with respect to the active site. The recombinant RNase A is another example of a strategy that may be used to develop a novel bioconjugate.

While the goal of isolating an RNase A sample that had been modified by SMPT at a single known site was not achieved, the methods developed to reach this conclusion may be used for other protein/chemical reagent systems that may have potential applications as a catalytically active bioconjugate. A base protein may be chosen that has fewer modifiable groups than RNase A (11 nucleophilic residues) along with a modification reagent that has a more limited specificity than the general reagent SMPT. The ability to incorporate a synthetic cofactor into the protein through either a chemically modified residue or a residue mutated by recombinant techniques represents a method for the development of other bioconjugates that may possess novel catalytic properties with potential therapeutic applications.

APPENDIX
EXPERIMENTAL PROCEDURE FOR MUTAGENESIS OF RIBONUCLEASE A F8C PERFORMED
BY LEE RALEY

PCR Mutagenesis

Polymerase chain reaction (PCR) primers containing the needed mutagenic sites were purchased from IDT Corporation (Coralville, IA).

5' primer with Nde I site	cat atg aaa gaa act gca gca gca aaa tgt gaa aga caa
3' primer with 2 TAG	cta cta cac aga tgc gtc aaa gtg aac cgg

The pET 23 RNase A vector (Novagen, Madison, WI) was added to a PCR tube containing 200 pmols of primers, 10 µL of buffer with $MgCl_2$, 0.3mM of deoxynucleotide triphosphates (DNTPs) (Promega, Madison, WI), and 5 units of Red Taq (Sigma, St. Louis, MO). Sterile water was added to 100 µL. A standard PCR procedure was followed without a hot start.

TOPO TA

A TOPO-TA Kit was purchased from Invitrogen (Carlsbad, CA). Two µL of PCR was added to one µL of TOPO-TA solution with 2 µL of sterile water. The mixture was left at room temperature for 5 minutes. Then it was placed on ice. TOPO10 cells (*Escherichia coli*) were transformed by the addition of β-mercaptoethanol (Sigma) followed by heat shock. The cells were incubated in a shaker at 37°C with SOC media (20 g Bacto-tryptone, 5g Bacto-yeast extract, .5g NaCl, 20mM glucose, Sigma) 1mL for 30 minutes and were then plated on Luria-Bertani (LB) agar with amp (Fisher, Springfield, NJ). Colonies were isolated and tested for insert by PCR. These colonies were then grown for plasmid isolation.

Amplification and Isolation of Double Stranded DNA

Double stranded plasmids were isolated using the Wizard Plus Minipreps DNA Purification System (Promega). The procedures suggested by the manufacturer were strictly followed. An overnight culture (ONC, 5 mL) was centrifuged (3800 rpm, 10 min, 4°C). The harvested cells were resuspended in cell suspension solution (400 μL) and transferred to an Eppendorf tube (Westbury, NY). Cell lysis solution (400 μL) was added and the tube inverted four times until the solution cleared. The cell debris was separated from the solution by centrifugation (micro centrifuge, 11000 rpm, 5 min, room temperature). Meanwhile, the syringe barrel was attached via the luer lock adapter to the mini column, which in turn was mounted on a vacuum manifold. Resuspended resin (1 mL) was pipetted into the column followed by the supernatant obtained above. The resin / lysate mixture was then drawn through the mini column by applying a vacuum. The vacuum was applied for another 30 s after all the solution had flown through. In all cases, column wash solution (2 mL) was drawn through, and the column was dried by applying the vacuum for 30 seconds. The mini column was removed from the barrel, transferred onto an Eppendorf tube and centrifuged (micro centrifuge, 11000 rpm, 2 min, room temperature). The minicolumn was transferred on another Eppendorf tube and filled with water (50 mL). Plasmid DNA was eluted by centrifuging (micro centrifuge, 11000 rpm, 20 s, room temperature). The mini column was discarded and the DNA concentration determined by UV-vis, λ_{260}.

Restriction Digest

Two ng of plasmid DNA was digested with Nde I and EcoRI restriction enzymes in EcoRI buffer (New England Biolabs, Beverly, MA). Two ng of DNA was placed in a PCR tube with 2 microliters of EcoRI buffer. Two µL of Nde I and EcoRI was added. Sterile water was added to 20 µL. The solution was allowed to incubate for four hours at room temperature.

Gel Purification

An agarose gel (1%) was prepared by mixing agarose (0.5 g) and 1 x tris acetic acid EDTA (TAE) buffer (50 mL) (Sigma). The suspension was heated in a microwave oven until the agarose was completely dissolved. Then, ethidium bromide (5 mL) (Sigma) was added, the solution (25 mL) poured into a gel electrophoresis chamber and was left to polymerize. 1 x TAE (50 mL) was used as running buffer. The samples were mixed with 5 x loading buffer and loaded on the agarose gel. The electrophoresis was run at 4 V / cm until the bromophenol blue (Sigma) bands reached the middle of the gel. Bands were isolated by detection with a UV / vis light followed by cutting from the gel. The band was purified using an agarose digest kit from Qiagen (Valencia, CA).

pET 23 B+ Ligation

The insert (0.400 ng) was added to a PCR tube along with 2 ng of pET 23 B+ DNA that had been predigested with Nde I and EcoRI. Two µL of T4 ligase buffer was added. 0.5 µL of T4 ligase (Promega) was added. Sterile water was added to 20 µL, followed by incubation at 4°C for 4 hours.

Transformation of Cells

Freshly prepared competent cells or electrocompetent cells (400 mL) were transformed with plasmid prepared as described above. Luria-Bertani ampicillin (LB-amp) medium (Difco, Detroit) (10 mL) was inoculated with one of the colonies obtained and incubated at 37°C, overnight. An aliquot (850 mL) was stored as a glycerol culture. The remainder of the ONC was used to prepare double stranded DNA, which was resequenced to exclude secondary mutations within the gene.

"Large Scale" Expression for pET23

For the expression of pET 23 B+, 5 2-L-Erlenmeyer flasks containing 800 mL Luria-Bertani* (LB*) (Difco, Detroit) medium were used. Ampicillin (400 µL), $MgSO_4$ (1 M, 800 µL) and $CaCl_2$ (1 M, 40 µL) were added to each flask. The medium was inoculated with 8 mL of the ONC and incubated at 30°C or 37°C for 4 hours until an optical density at 550 nm of 1.0 was reached. The expression was initiated by addition of 0.4mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma). After four hours of incubation the cells were centrifuged (G3, 5000 rpm, 5 min, 4°C) and the pellet stored at -80°C overnight

Cell Lysis with "French Press"

The cells were resuspended in French press buffer (four times the pellet's weight) containing Tris-HCl (50 mM, pH 7.6), NaCl (100 mM), EDTA (1 mM, pH 8), phenylmethylsulphyl flouride (PMSF) (1 mM) and NaN_3 (0.02%) and lysed at 1000 psi at room temperature. The resulting solution was centrifuged (G3, 7000 rpm, 30 min, 4°C) and the supernatant discarded. The pellet was weighed and either immediately resuspended or stored at -80°C.

Denaturation of "Inclusion Bodies"

The pellet obtained previously was resuspended in a buffer (ten times the pellet's wet weight) consisting of urea (10 M), 2-mercaptoethanol (500 mM), NH_4OAc (20 mM, pH 6.8), PMSF (1 mM), sarcosine (10 mM) and NaN_3 (0.02%). The suspension was stirred and vortexed at room temperature until the cell debris was completely dissolved, followed by shaking at 30°C for another 30 min. After centrifugation (7000 rpm, 30 min., 20°C) the pellet was discarded and the supernatant applied to an equilibrated carboxymethyl (CM) cationic exchange column.

CM Column

A CM exchange column (15 cm, 3 cm) was prepared with CM Bio-Gel (15 mL) (Bio-Rad, Hercules, CA) and equilibrated twice with NH_4OAc (20 mM, pH 7, 50 mL). The conductivity of the buffer was measured before and after the chromatography and was found to be equal. The supernatant was applied to the equilibrated column and washed with NH_4OAc (20 mM, pH 7, 50 mL). The proteins were eluted with a solution (250 mL) containing NaCl (0.5 M) and NH_4OAc (20 mM, pH 7). The eluted proteins were refolded overnight in a glutathione buffer (10 mM GSH / 1 mM GSSG) (Sigma) that had been adjusted to pH 8. All eluates were checked for RNase activity by UpA assays. A slight activity was detected in the flow-through suggesting that the RNase did not completely bind under the applied conditions.

Ultrafiltration of Protein Solutions

The eluates containing active RNase obtained from the CM column were concentrated with an Amicon concentrator (3.1 bar) equipped with a YM3 (MW cutoff = 3500) membrane. The buffer was changed twice to sodium acetate (NaOAc) (50 mM, pH 5, 0.02% NaN_3, 50 mL) and the solution concentrated to a final volume of 5 mL.

pUp-Agarose Column

One batch of uridine 2',5'-and 3',5'-diphosphate-agarose (pUp) (325 mg) was soaked in NaOAc (50 mM, pH 5, 5 mL) at 4°C overnight. The suspension (2.5mL) was poured into an Econo column (10 cm x 1 cm) (Fisher, Springfield, NJ) and equilibrated with NaOAc (50 mM, pH 5, 10 mL). The concentrated solution obtained containing active RNase A was applied to the column. The column was washed with NaOAc (50mM, pH

5, 50 mL) and the protein eluted three times with a NaOAc buffer (50 mM, pH 5, 10 mL) containing NaCl (0.5 M, 1 M and 2.4 M, respectively). The eluates were checked for RNase activity.

REFERENCES

1. Means, G. E., and Feeney, R. E. (1998) Chemical Modifications of Proteins: A Review. *J. Food Biochem.* **22**, 399-426.

2. Horton, H. R., Moran, L. A., Ochs, R. S., Rawn, J. D., and Scrimgeour, K. G. (1993) Principles of Biochemistry, 3-4 - 3-24. Englewood Cliffs, Neil Patterson Publishers / Prentice Hall, Inc.

3. Campbell, N. A. (1987) Biology. California, The Benjamin / Cummings Publishing Company, Inc.

4. Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H. (1948) Essential Groups for the Interaction of Ovomuciod (Egg White Trypsin Inhibitor) and Trypsin, and for Tryptic Activity. *J. Biol. Chem.* **177**, 385-402.

5. Fraenkel-Conrat, H., and Feeney, R. E. (1950) The Metal-Binding Activity of Conalbumin. *Arch. Biochem.* **29**, 101-113.

6. Wofsy, L., Metzger, H., and Singer, S. J. (1962) Affinity Labeling-A General Method for Labeling the Active Sites of Antibody and Enzyme Molecules. *Biochemistry.* **1**, 1031-1039.

7. Fruton, J. S. (1992) An Episode in the History of Protein Chemistry - Edman, Pehr: Method for the Sequential Degradation of Peptides. *Int. J. Pep. Prot. Res.* **39**, 189-194.

8. Edman, P. (1950) Method for Determination of the Amino Acid Sequence in Peptides. *Acta. Chem. Scand.* **4**, 283-293.

9. Edman, P., and Begg, G. (1967) A Protein Sequenator. *Eur. J. Biochem.* **1**, 80-91.

10. Horton, H. R., Moran, L. A., Ochs, R. S., Rawn, J. D., and Scrimgeour, K. G. (1993) Principles of Biochemistry, 3-19-3-22. Englewood Cliffs, Neil Patterson Publishers / Prentice Hall, Inc.

11. Hirs, C. H. W., and Timasheff, S. N. (1972) Methods in Enzymology. 387-651. New York, Academic Press. Colowick, S. P. and Kaplan, N. O, Eds.

12. Hirs, C. H. W., and Timasheff, S. N. (1972) Methods in Enzymology. 655-671. New York, Academic Press. Colowick, S. P. and Kaplan, N. O, Eds.

13. Means, G. E., and Feeney, R. E. (1971) Affinity Labeling of Pancreatic Ribonuclease. *J. Biol. Chem.* **246**, 5532-5533.

14. Jelenc, P. C., Cantor, C. R., and Simon, S. R. (1978) High Yield Photoreagents for Protein Crosslinking and Affinity Labeling. *Proc. Natl. Acad. Sci. USA.* **75, No. 8**, 3564-3568.

15. Klein, G., Satre, M., Dianoux, A., and Vignais, P. V. (1980) Radiolabeling of Natural Adenosine Triphosphatase Inhibitor with Phenyl (^{14}C) Isothiocyanate and Study of its Interction with Mitochondrial Adenosine Triphosphatase. Localization of Inhibitor Binding Sites and Stoichiometry of Binding. *Biochemistry.* **19**, 2919-2925.

16. Genchi, G., Petrone, G., De Palma, A., Cambria, A., and Palmieri, F. (1988) Thiocyanates with the Mitochondrial Phosphate Carrier. I. Covalent Modification and Inhibition of Phosphate Transport. *Biochim. Biophys. Acta.* **936**, 413-420.

17. Fonyo, A., Ligeti, E., Palmieri, F., and Quagliariello, E. (1975) Biomembranes, Structure and Function. 287-306. Budapest and Amsterdam, Akademiai Kiado and Elsevier.

18. Staros, J. V. (1988) Membrane-Impermeant Cross-Linking Reagents: Probes of the Structure and Dynamics of Membrane Proteins. *Acc. Chem. Res.* **21**, 435-441.

19. Ebina, S., Lerner, R. A., and Wright, P. E. (1989) Chemical Modifications of Bovine Pancreatic Trypsin Inhibitor for Single Site Coupling of Immunogenic Peptides for NMR Conformational Analysis. *J. Biol. Chem.* **264**, 7882-7888.

20. Chang, G. G., Shiao, M. S., Lee, K. R, and Wu, J. J. (1990) Modification of Human Placental Alkaline Phosphatase by Periodate-Oxidized 1,N^6-Ethenoadenosine Monophosphate. *Biochem. J.* **272**, 683-690.

21. Stigbrand, T., and Fishman, W. H. (1984) Human Alkaline Phosphatases. 79-135. New York, Alan R. Liss.

22. Bostrom, B., Hogenkamp, H. P. C., and Mirkin, B. L. (1988) Inhibitory Effect of Adenosine Ddialdehyde on *Insitu* Murine Neuro-Blastom Growth. *Cancer Res.* **48**, 5933-5936.

23. Mirkin, B. L., Odea, R. F., and Hogenkamp, H. P. (1987) Adenosine Nucleoside and Dialdehyde Analogs on Murine Neuroblastoma in Tissue-Culture - Structure-Activity - Relationships. *Cancer Res.* **47**, 3650-3655.

24. Gullick, W. J. (1986) Peptide Mapping of Proteins. 207-225. New York. John Wiley & Sons Ltd.

25. LaDine, J. R., and Cross, R. L. (1991) The Adenine Nucleotide-Binding Site in Yeast 3-Phosphoglycerate Kinase. *J. Biol. Chem.* **266, No. 11**, 7194-7198.

26. Siuzdak, G. (1996) Mass Spectrometry for Biotechnology. **1**. San Diego, Academic Press.

27. Skoog, D. A., and Leary, J. J. (1992) Mass Spectrometry. **4**, 420-459. Fort Worth, Philadelphia, San Diego, New York, Sanders College Publishing / Harcourt Brace College Publishers.

28. Larsen, B.S., and McEwen, C.N., Eds. (1998) Mass Spectrometry of Biological Molecules. **2**. New York, Marcel Dekker.

29. Beuhler, R. J., Flanigan, E., Green, L. J., and Friedman, L. (1974) Proton Transfer Mass Spectrometry of Peptides, A Rapid Heating Technique for Underivatized Peptides Containing Arginine. *J. Am. Chem. Soc.* **96**, 3990.

30. Barber, M., Bordioli, R. S., and Elliot, G. J. (1984) Modern Soft Ionization Sources. *Gazz. Chim. Ital.* **114**, 305-311.

31. Roepstorff, P., Nielsen, P. F., Klarskov, K., and Hojrup, P. (1988) Applications of Plasma Desorption Mass Spectrometry in Peptide and Protein Chemistry. *Biomed. Environ. Mass Spectrom.* **16**, 9-18.

32. Clench, M. R., Garner, G. V., Gordon, D. B., and Barber, M. (1985) Surface Effects in FAB Mapping of Proteins and Peptides. *Biomed. Mass Spectrom.* **12**, 355-367.

33. Barber, M., Bordoli, R. S., Elliot, R. D., Sedgwick, R. D., Tyler, A. N., and Green, B. N. (1982) Fast Atom Bombardment Mass-Spectrometry of Bovine Insulin and Other Large Peptides. *J. Chem. Soc. Chem. Commun.* 936-938.

34. Beckey, H. D. (1977) Principles of Field Ionization and Field Desorption Mass Spectrometry. Oxford, Pergamon.

35. Simons, D. S., Colby, D. N., and Evans Jr, C. A. (1974) Electrohydrodynamic Ionization Mass-Spectrometry - The Ionization of Liquid Glycerol and Non-Volatile Organic Solutes. *Int. J. Mass Spectrom. Ion Phys.* **15**, 291-302.

36. Cook, K. D. (1986) Electrohydrodynamic Mass-Spectrometry. *Mass Spectrom. Rev.* **5**, 467.

37. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989) Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *Science.* **246**, 64-71.

38. Favretto, D., Seraglia, R., Traldi, P., Curcuruto, O., and Hamdan, M. (1994) Electrospray vs. Matrix-Assisted Laser Desorption / Ionization. 1. Some Examples in the Protein Field. *Org. Mass Spectrom.* **29**, 526-532.

39. Loo, J. A. (1995) Bioanalytical Mass Spectrometry: Many Flavors to Choose. *Bioconjugate Chem.* **6**, 644-665.

40. Kussmann, M., and Roepstorff, P. (1998) Characterisation of the Covalent Structure of Proteins from Biological Material by MALDI Mass Spectrometry - Possibilities and Limitations. *Spectrosc.-Int. J.* **14**, 1-27.

41. Arnott, D., Shabanowitz, J., and Hunt, D. F. (1993) Proteins and Peptides: Sensitive and Accurate Mass Measurement and Sequence Analysis. *Clinical Chem.* **39**, 2005-2010.

42. Dole, M., Mach, M. M., Hines, R. L., Mobley, R. C., Ferguson, L. P., and Alice, M. B. (1968) Molecular Beams of Macroions. *J. Chem. Phys.* **49**, 2240-2249.

43. Mach, M. M., Kralick, P., Rheude, A., and Dole, M. (1970) Molecular Beams of Macroions. II. *J. Chem. Phys.* **52**, 4977-4986.

44. Bruins, A. P. (1998) Mechanistic Aspects of Electrospray Ionization. *J. Chromatogr. A.* **794**, 345-357.

45. Przybylski, M., and Glocker, M. O. (1996) Electrospray Mass Spectrometry of Biomacromolecular Complexes with Noncovalent Interactions - New Analytical Perspectives for Supramolecular Chemistry and Molecular Recognition Processes. *Angew. Chem. Int. Ed.* **35** , 807-826.

46. Kebarle, P., and Ho, Y. (1997) Electrospray Ionization Mass Spectrometry. New York, John Wiley & Sons Ltd.

47. Kebarle, P., and Tang, L. (1993) From Ions in Solution to Ions in the Gas-Phase - The Mechanism of Electrospray Mass-Spectrometry. *Anal. Chem.* **65**, 972A-986A.

48. Fenn, J. B. (1993) Ion Formation from Charged Droplets: Roles of Geometry, Energy, and Time. *J. Am. Soc. Mass. Spectrom.* **4**, 524-535.

49. Ashton, D. S., Beddell, C. R., Cooper, D. J., Green, B. N., and Oliver, R. W. A. (1993) Mechanism and Production of Ions in Electrospray Mass Spectrometry. *Org. Mass Spectrom.* **28** , 721-728.

50. Iribarne, J. V., and Thompson, B. A. (1976) On Evaporation of Small Ions from Charged Droplets. *J. Chem. Phys.* **64**, 2287-2294.

51. Thompson, B. A., and Iribarne, J. V. (1979) Field-Induced Ion Evaporation from Liquid Surfaces at Atmospheric Pressure. *J. Chem. Phys.* **71**, 4451-4463.

52. Iribarne, J. V., Dziedzic, P. J., and Thompson, B. A. (1983) Atmospheric-Pressure Ion Evaporation Mass-Spectrometry. *Int. J. Mass Spectrom. Ion Phys.* **50**, 331-347.

53. Schmelzeisen-Redeker, G., Bütfering, L., and Röllgen, F. W. (1989) Desolvation of Ions and Molecules in Thermospray Mass-Spectrometry. *Int. J. Mass Spectrom. Ion Processes.* **90**, 139-150.

54. Tang, L., and Kebarle, P. (1993) Dependence of Ion Intensity in Electrospray Mass-Spectrometry on the Concentration of the Analytes in the Electrosprayed Solution. *Anal. Chem.* **65**, 3654-3668.

55. Schnier, P. D., Gross, D. S., and Williams, E. R. (1995) On the Maximum Charge State and Proton Transfer Reactivity of Peptide and Protein Ions Formed by Electrospray Ionization. *J. Am. Soc. Mass. Spectrom.* **6**, 1086-1097.

56. Bleicher, K., and Bayer, E. (1994) Various Factors Influencing the Signal Intensity of Oligonucleotides in Electrospray Mass-Spectrometry. *Biol. Mass S.* **23**, 320-322.

57. Gelpi, E. (5-26-1995) Biomedical and Biochemical Applications of Liquid-Chromatography Mass-Spectrometry. *J. Chromatogr. A.* **703**, 59-80.

58. Mehlis, B., and Kertscher, U. (10-10-1997) Liquid Chromatography Mass Spectrometry of Peptides of Biological Molecules. *Anal. Chim. Acta.* **352**, 71-83.

59. Dass, C. (1999) Recent Developments and Applications of High-Performance Chromatography Electrospray Ionization Mass Spectrometry. *Curr. Org. Chem.* **3**, 193-209.

60. Hunt, D. F., Alexander, J. E., McCormack, P. A., Martino, P. A., Michel, H., Shabanowitz, J., and Sherman, N. (1991) Mass Spectrometric Methods for Protein and Peptide Sequence Analysis. 441-454. San Diego, Academic Press, Inc.

61. Yates, J. R. III, Eng, J. K., McCormack, A. L., and Schieltz, D. (1995) Method to Correlate Tandem Mass Spectra of Modified Peptides to Amino Acid Sequences in the Protein Database. *Anal. Chem.* **67**, 1426-1436.

62. Eng, J. K., McCormack, A. L., and Yates, J. R. III. (1994) An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. *J. Am. Soc. Mass. Spectrom.* **5**, 976-989.

63. Jaffe, H., Veeranna, and Pant, H. C. (1998) Characterization of Serine and Threonine Phosphorylation Sites in β-Elimination/ Ethanethiol Addition-Modified Proteins by Electrospray Tandem Mass Spectrometry and Database Searching. *Biochemistry.* **37**, 16211-16224.

64. Cottrell, J. S. (1994) Protein Identification by Peptide Mass Fingerprinting. *Peptide Res.* **7**, 115-&.

65. Covey, T. (1996) Liquid Chromatography/Mass Spectrometry for the Analysis of Protein Digests. **1**, 83-99. Towowa, NJ, Humana Press Inc. Methods in Molecular Biology. Walker, J. M.

66. Hunt, D. F., Henderson, R. A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A. L., Appella, E., and Engelhard, V. H. (1992) Characterization of

Peptides Bound to the Class I MHC Molecule HLA-A2.1 by Mass Spectrometry. *Science.* **255**, 1261-1263.

67. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F., and Engelhard, V. H. (1992) HLA-A2.1 - Associated Peptides from Mutant Cell Line: A Second Pathway of Antigen Presentation. *Science.* **255**, 1264-1266.

68. Tracey, B. M., Feizi, T., Abbott, M., Carruthers, R. A., Green, B. N., and Lawson, A. M. (1992) Subunit Molecular Mass Assignment of 14,654 to the Soluble β-Galactoside-Binding Lectin from Bovine Heart Muscle and Demonstration of Intramolecular Disulfide Bonding Associated with Oxidative Inactivation. *J. Biol. Chem.* **267**, 10342-10347.

69. Ball, L. E., Oatis, J. E., Dharmasiri, K., Busman, M, Wang, J. Y., Cowden, L. B., Galijatovic, A., Chen, N., Crouch, R. K., and Knapp, D. R. (1999) Mass Spectrometric Analysis of Integral Membrane Proteins: Application to Complete Mapping of Bacteriorhodopsins and Rhodopsin. *Protein Sci.* **7**, 758-764.

70. Raida, M., Schulz-Knappe, P., Heine, G., and Forssmann, W. (1999) Liquid Chromatography and Electrospray Mass Spectrometric Mapping of Peptides from Human Plasma Filtrate. *J. Am.Soc. Mass. Spectrom.* **10**, 45-54.

71. Jungblut, P., Otto, A., Zeindl-Eberhardt, E., Pleissner, K. P., Kreckt, M., Regitz-Zagrosek, V., Fleck, E., and Wittmann-Liebold, B. (1994) Protein-Composition of the Human Heart - The Construction of a Myocardial 2-Dimensional Electrophoresis Database. *Electrophoresis.* **15**, 685-707.

72. Jungblut, P., Thiede, B., Zimny-Arndt, U., Müller, E. C., Scheler, C., Wittmann-Liebold, B., and Otto, A. (1996) Resolution Power of Two-Dimensional Electrophoresis and Identification of Proteins from Gels. *Electrophoresis.* **17**, 839-847.

73. Gevaert, K., Verschelde, J. L., Puype, M., Van Damme, J., Goethals, M, De Boeck, S., and Vanderkerckhove, J. (1996) Structural Analysis and Identification of Gel-Purified Proteins, Available in the Femtomole Range, Using a Novel Computer Program for Peptide Sequence Assignment by Matrix-Assisted Laser Desorption Ionization Reflectron Time-of-Flight Mass Spectrometry. *Electrophoresis.* **17**, 918-924.

74. Akashi, S., Shirouzu, M., Terada, T., Ito, Y., Yokoyama, S., and Takio, K. (1997) Characterization of the Structural Difference between Active and Inactive Forms of the Ras Protein by Chemical Modification Followed by Mass Spectrometric Peptide Mapping. *Anal. Biochem.* **248**, 15-25.

75. Zappacosta, F., Ingallinells, P., Scaloni, A, Pessi, A., Bianchi, E., Sollazzo, M., Tramontano, A., Marino, G., and Pucci, P. (1997) Surface Topology of Minibody by Selective Chemical Modifications and Mass Spectrometry. *Protein Sci.* **6**, 1901-1909.

76. Wood, T. D., Guan, Z. Q., Borders, C. L., Chen, L. H., Kenyon, G. L., and McLafferty, F. W. (1998) Creatine Kinase: Essential Arginine Residues at the Nucleotide Binding Site Identified by Chemical Modification and High-Resolution Mass Spectrometry. *Proc. Natl. Acad. Sci. USA*. **95**, 3362-3365.

77. Meares, C. L. (1990) Introduction to Bioconjugate Chemistsry. *Bioconjugate Chem.* **1**, 1.

78. Levine, H. L., Nakagawa, Y., and Kaiser, E. T. (1977) Flavopapain: Synthesis and Properties of Semi-Synthetic Enzymes . *Biochem. Biophys. Res. Commun.* **76**, 64-70.

79. Kaiser, E. T., and Lawrence, D. S. (1984) Chemical Mutation of Enzymes. *Science*. **226**, 505-511.

80. Pan, L. P., Durham, B., Wolinska, J., and Millett, F. (1988) Preparation and Characterization of Singly Labeled Ruthenium Polypyridine Cytochrome *c* Derivatives. *Biochemistry*. **27**, 7180-7184.

81. Albert, D. E., Douglas, M. B., Hintz, M. A., Youngen, C. S., and Keyes, M. H. (1992) Semisynthetic Fluorohydrolases Prepared by Chemical Modification of Ribonuclease. *Enzyme Microb. Technol.* **14**, 885-892.

82. Distefano, M. D., Kuang, H., Qi, D., and Mazhary, A. (1998) The Design of Protein-Based Catalysts Using Semisynthetic Methods. *Curr. Opin. Struc. Biol.* **8**, 459-465.

83. Ebright, R. H., Ebright, Y. W., Pendergrast, P. S., and Gunasekera, A. (1990) Conversion of a Helix-Turn-Helix Motif Sequence -Specific DNA Binding Protein into a Site-Specific DNA Cleavage Agent. *Proc. Natl. Acad. Sci.USA*. **87**, 2882-2886.

84. Sigman, D. S., Bruice, T. C., Mazumder, A., and Sutton, C. L. (1993) Targeted Chemical Nucleases. *Acc. Chem. Res.* **26**, 98-104.

85. Sigman, D. S., Mazumder, A., and Perrin, D. M. (1997) Chemical Nucleases. *Chem. Rev.* **93**, 2295-2316.

86. Landgraf, R., Pan, C., Sutton, C. L., Pearson, L., and Sigman, D. S. (1996) Engineering of DNA Binding Proteins Into Site-Specific Cutters: Reactivity of Trp Repressor-1,10-Phenanthroline Chimeras. *Protein Engineering*. **9**, 603-610.

87. Chen, C. B., Mazumder, A., Constant, J., and Sigman, D. S. (1993) Nuclease Activity of 1, 10-Phenanthroline-Copper. New Conjugates with Low Molecular Weight Targeting Ligands. *Bioconjugate Chem.* **4**, 69-77.

88. Sigman, D. S., Kuwabara, M. D., Chen, C. B., and Bruice, T. C. (1991) Nuclease Activity of 1,10-Phenanthroline-Copper in Study of Protein-DNA Interactions. **1**, 414-433. New York, Academic Press, Inc.

89. Hoyer, D., Cho, H., and Schultz, P. G. (1990) A New Strategy for Selective Protein Cleavage. *J. Am. Chem. Soc.* **112**, 3249-3250.

90. Zuckermann, R. N., and Schultz, P. G. (1988) A Hybrid Sequence-Selective Ribonuclease S. *J. Am. Chem. Soc.* **110**, 6592-6594.

91. Mack, D. P., and Dervan, P. B. (1990) Nickel-Mediated Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein. *J. Am. Chem. Soc.* **112**, 4604-4606.

92. Mack, D. P., and Dervan, P. B. (1992) Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein, Ni(II)GGH (Hin 139-190). *Biochemistry.* **31**, 9399-9405.

93. Davies, R. R., and Distefano, M. D. (1997) A Semisynthetic Metalloenzyme Based on a Protein Cavity That Catalyzes the Enantioselective Hydrolysis of Ester and Amide Substrates. *J. Am. Chem. Soc.* **119**, 11643-11652.

94. Anfinsen, C. B., and White, F. H. (1960) The Ribonucleases: Occurence, Structure and Properties. Methods in Enzymology. 95-122.

95. Raines, R. T. (1998) Ribonuclease A. *Chem. Rev.* **98**, 1045-1065.

96. D' Alessio, G., and Riordan, J. F. (1997) Ribonucleases: Stuctures and Functions. **1**, 271-304. San Diego, Academic Press.

97. Jones, W. (1920) *Am. J. Physiol.* **52**, 203.

98. Kunitz, M. (1939) Isolation from Beef Pancreas of a Crystalline Protein Possessing Ribonuclease Activity. *Science.* **90**, 112-115.

99. Kunitz, M. (1946) A Spectrophotometric Method for the Measurement of Ribonuclease Activity. *J. Biol. Chem.* 563-568.

100. Hirs, C. H. W., Moore, S., and Stein, W. H. (1952) A Chromatographic Investigation of Pancreatic Ribonuclease. Methods in Enzymology. 493-506.

101. Crestfield, A. M., Stein, W. H., and Moore, S. (1963) On the Preparation of Bovine Pancreatic RNase A. *J. Biol. Chem.* **238**, 618-621.

102. Crook, E. M., Mathias, A. P., and Rabin, B. R. (1960) Spectrophotometric Assay of Bovine Pancreatic Ribonuclease by the use of Cytidine 2',3'-phosphate. *Biochem. J.* **74**, 234-238.

103. Lazzari, E. P., Sullivan, E., and Murray, D. R. (1970) A Sensitive Colorimetric Assay for Ribonuclease. *Texas Reports on Biology and Medicine.* **28**, 561-564.

104. Glick, D. M., and Barnard, E. A. (1970) Some Relations of Lysine Residues to Reactivies in Pancreatic Ribonuclease. *Biochim. Biophys. Acta.* **214**, 326-342.

105. Patthy, L., and Smith, E. L. (1975) Identification of Functional Arginine Residues in Ribonuclease A and Lysozyme. *J. Biol. Chem.* **250, No. 2**, 565-569.

106. Kartha, G., Bello, J., and Harker, D. (1967) Tertiary Structure of Ribonuclease. *Nature.* 862-865.

107. Carlisle, C. H., Palmer, R. A., Mazumdar, S. K., Gorinsky, B. A., and Yeates, D. G. R. (1974) The Structure of Ribonuclease at 2.5 Angstrom Resolution. *J. Mol. Biol.* **85**, 1-18.

108. Wlodawer, A., Svensson, L. A., Sjolin, L., and Gilliland, G. L. (1988) Structure of Phosphate-Free Ribonuclease A Refined at 1.26A. *Biochemistry.* **27**, 2705-2717.

109. Kim, E. E., Varadarajan, R., Wyckoff, H. W., and Richards, F. M. (1992) Refinement of the Crystal Structure of Ribonuclease S. Comparison with and between the Various Ribonuclease A Structures. *Biochemistry.* **31**, 12304-12314.

110. Arnold, U., and Ulrich-Hofmann, R. (1997) Kinetic and Thermodynamic Thermal Stabilities of Ribonuclease A and Ribonuclease B. *Biochemistry.* **36**, 2166-2172.

111. Matthew, J. B., and Richards, F. M. (1982) Anion Binding and pH-Dependent Electrostatic Effects in Ribonuclease. *Biochemistry.* **21**, 4989-4999.

112. Silverman, D. N., Kotelchuck, D., Taylor, G. T., and Scheraga, H. A. (1972) Nuclear Magnetic Resonance Study of the N-Terminal Fragment of Bovine Pancreatic Ribonuclease. *Arch. Biochem. Biophys.* **150**, 757-766.

113. Brown, L. R., and Bradbury, J. H. (1975) Proton-Magnetic-Resonance Structures of the Lysine Residues of Ribonuclease A. *Eur. J. Biochem.* **54**, 219-227.

114. Ihnat, M. (1972) Binding of Copper (II) by Ribonuclease A and Ribonuclease S-Peptide Investigated by H Nuclear Magnetic Resonance Spectroscopy. *Nuclear Magnetic Resonance.* **11**, 3483-3492.

115. Means, G. E., and Feeney, R. E. (1971) Affinity Labeling of Pancreatic Ribonuclease. *J. Biol. Chem.* **246**, 5532-5533.

116. Bello, J., and Nowoswiat, E. F. (1971) Multiple Carboxymethylation of Histidines in Bovine Ribonuclease A. *Eur. J. Biochem.* **22**, 225-234.

117. Patthy, L., and Smith, E. L. (1975) Reversible Modification of Arginine Residues. *J. Biol. Chem.* **250, No. 2**, 557-564.

118. Blackburn, P., and Jailkhani, B. L. (1979) Ribonuclease Inhibitor from Human Placenta: Interaction with Derivatives of Ribonuclease A. *J. Biol. Chem.* **254**, 12488-12493.

119. Blackburn, P., and Gavilanes, J. G. (1982) Identification of Lysine Residues in the Binding Domain of Ribonuclease A for the RNase Inhibitor from Human Placenta. *J. Biol. Chem.* **257, No. 1**, 316-321.

120. Watkins, N. G., Thorpe, S. R., and Baynes, J. W. (1985) Glycation of Amino Groups in Protein. *J. Biol. Chem.* **260, No. 19**, 10629-10636.

121. Alonso, J., Nogues, V., and Cuchillo, C. M. (1986) Modification of Bovine Pancreatic Ribonuclease A with 6-Chloropurine Riboside. *Arch. Biochem. Biophys.* **246**, 681-689.

122. Pares, X., Llorens, R., Arus, C., and Cuchillo, C. M. (1980) The reaction of Bovine Pancreatic Ribonuclease A with 6-Chloropurineriboside 5'-Monophosphate. *Eur. J. Biochem.* **105**, 571-579.

123. Camilleri, P., Haskins, N. J., Rudd, P. M., and Saunders, M. R. (1993) Applications of Electrospray Mass Spectrometry to Studies on the Structural Properties of Ribonuclease A and Ribonuclease B. *Rapid Commun. Mass Spectrom.* **7**, 332-335.

124. Raverdino, V., Lapolla, A., Fedele, D., Baldo, L., Aronica, R., Catinella, S., Seraglia, R., and Traldi, P. (1996) An Electrospray Investigation on *in vitro* Glycation of Ribonuclease. *Rapid Commun. Mass Spectrom.* **10**, 178-182.

125. Bruce, J. E., Hofstadler, S. A., Winger, B. E., and Smith, R. D. (1994) Characterization of Ribonuclease B Heterogeneity and the Identification and Removal of Phosphate Adducts by High Resolution Electrospray Ionization Fourier Transfrom Ion Cyclotron Resonance Mass Spectrometry. *Int. J. Mass Spectrom. Ion Processes.* **132**, 97-107.

126. Camilleri, P., and Haskins, N. J. (1993) Investigating the Non-covalent Interaction of Cytidylic Acids, with Ribonuclease A by Electrospray Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **7**, 603-604.

127. Neumann, U., and Hofsteenge, J. (1994) Interaction of Semisynthetic Variants of RNase A with Ribonuclease Inhibitor. *Protein Sci.* **3**, 248-256.

128. Goodlett, D. R., Ogorzalek Loo, R. R., Loo, J. A., Wahl, J. H., Udseth, H. R., and Smith, R. D. (1994) A Study of the Thermal Denaturation of Ribonuclease S by Electrospray Ionization Mass Spectrometry. *J. Am. Soc. Mass. Spectrom.* **5**, 614-622.

129. Alexandrescu, A. T., Rathgeb-Szabo, K., Rumpel, K., Jahnke, W., Schulthess, T., and Kammerer, R. A. (1998) ^{15}N Backbone Dynamics of the S-Peptide from Ribonuclease A in its Free and S-Protein Bound Forms: Toward a Site-Specific Analysis of Entropy Changes Upon Folding. *Protein Sci.* **7**, 389-402.

130. Ogorzalek Loo, R. R., Goodlett, D. R., Smith, R. D., and Loo, J. A. (1993) Observation of a Noncovalent Ribonuclease S-Protein/S-Peptide Complex by Electrospray Ionization Mass Spectrometry. *J. Am. Chem. Soc.* **115**, 4391-4392.

131. Glocker, M. O., Borchers, C., Fiedler, W., Suckau, D., and Przybylski, M. (1994) Molecular Characterization of Surface Topology in Protein Tertiary Structures by

Amino-Acylation and Mass Spectrometric Peptide Mapping. *Bioconjugate Chem.* **5**, 583-590.

132. Jue, R., Lambert, J. M., Pierce, L. R., and Traut, R. R. (1978) Addition of Sulfhydryl Goups to *Escherichia coli* Ribosomes by Protein Modification with 2-Iminothiolane. *Biochemistry.* **17**, 5399-5406.

133. Lindsay, D. G., and Shall, S. (1971) The Acetylation of Insulin. *Biochem. J.* **121**, 737-745.

134. Schnell, R., Vietta, E., Schindler, J., Barth, S., Winkler, U., Borchmann, P., Hansmann, M. L., Diehl, V., Ghetie, V., and Engert, A. (1998) Clinical Trials with an Anti-CD25 Ricin A-Chain Experimental and Immunotoxin (RFT5-SMPT-dgA) in Hodgkin's Lymphoma. *Leuk. Lymphoma.* **30**, 525.

135. Engert, A., Schnell, R., Radszuhn, A., Hatwig, M. T., Drillich, S, Schon, G., Tesch, H., Hansmann, M. L , Barth, S., Schindler, J., Ghetie, V., Uhr, J, and Vietta, E. (1997) A Phase-I Study of an Anti-CD25 Ricin A-Chain Immunotoxin (RFT5-SMPT-dgA) in Patients with Refractory Hodgkin's Lymphoma. *Blood.* **89**, 403-410.

136. Ghetie, V., Thorpe, P., Ghetie, M. A., Knowles, P., Uhr, J., and Vietta, E. (1991) The GLP Large-Scale Preparation of Immunotoxins Containing Deglycosylated Ricin-A Chain and a Hindered Disulfide Bond. *J. Immun.* **142**, 223-230.

137. Sanchez, E. F., Bush, L. R., Swenson, S., and Markland, F. S. (1997) Covalent Attachment of an RGD-Like Peptide to Create a Potentially More Effective Thrombol.S. Chimeric Fibrolase: Covalent Attachment of an RGD-Like Peptide to Create a Potentially More Effective Thrombolytic Agent. *Thromb. Res.* **87**, 289-302.

138. Eftink, M. R., and Biltonen, R. L. (1983) Energetics of RNase A Catalysis. 3. Temperature Dependence of the Hydrolysis of Cytidine Cyclic 2',3'-Phosphate. *Biochemistry.* **22**, 5140-5150.

139. Wigger, M., Nawrocki, C. H., Watson, J. R., Eyler, J. R., and Benner, S. A. (1997) Assessing Enzyme Substrate Specificity Using Combinatorial Libraries and Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **11**, 1749-1752.

140. Lang, G. H. L. (1998) Studies of Organometallic and Biological Systems with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *University of Florida, Ph.D.Dissertation.*

141. Pan, L. P., Durham, B., Wolinska, J., and Millett, F. (1988) Preparation and Characterization of Singly Labeled Ruthenium Polypyridine Cytochrome *c* Derivatives. *Biochemistry.* **27**, 7180-7184.

142. Wilkinson, J. M. (1986) Fragmentation of Polypeptides by Enzymic Methods. 121-135. New York, John Wiley & Sons.

143. Horton, H. R., Moran, L. A., Ochs, R. S., Rawn, J. D., and Scrimgeour, K. G. (1993) Principles of Biochemistry. 5-18 - 6-26. Englewood Cliffs, Neil Patterson Publishers/Prentice Hall, Inc.

144. Glocker, M. O., Borchers, C., Fiedler, W., Suckau, D., and Przybylski, M. (1994) Molecular Characterization of Surface Topology in Protein Tertiary Structures by Amino-Acylation and Mass Spectrometric Peptide Mapping. *Bioconjugate Chem.* **5**, 583-590.

145. Marshall, A. G., Hendrickson, C. L., and Jackson, G. S. (1998) Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: A Primer. *Mass Spectrom. Rev.* **17**, 1-35.

146. Halliday, D., Resnick, R., and Walker, J. (1993) Fundamentals of Physics, 4th edition, 487-489. New York, John Wiley & Sons, Inc.

147. Marshall, A. G. (1985) Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Acc. Chem. Res.* **18**, 316-322.

148. Comisarow, M. B., and Marshall, A. G. (1996) The Early Development of Fourier Transform Ion Cyclotron Resonance (FT-ICR) Spectroscopy. *J. Mass Spec.* **31**, 581-585.

149. Wigger, M., Nawrocki, C. H., Watson, J. R., Eyler, J. R., and Benner, S. A. (1997) Assessing Enzyme Substrate Specificity Using Combinatorial Libraries and Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **11**, 1749-1752.

150. Lang, G. H. L. (1998) Studies of Organometallic and Biological Systems with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *University of Florida, Ph.D.Dissertation.*

151. Liang, Z., and Marshall, A. G. (1990) Precise Relative Ion Abundances from Fourier Transform Ion Cyclotron Resonance Magnitude-Mode Mass Spectra. *Anal. Chem.* **62**, 70-75.

152. Marshall, A. G., and Verdun, F. R. (1989) Fourier Transforms in Optical, NMR, and Mass Spectroscopy. **1**. Amsterdam, Elsevier.

153. Gordon, E. F., and Muddiman, D. C. (1999) Relative Ion Abundance Measurements of Electrosprayed Biological Molecules from FTICR Mass Spectra. *47th ASMS Conference on Mass Spectrometry and Allied Topics.*

154. Fenn, J. B. (1993) Ion Formation From Charged Droplets: Roles of Geometry, Energy, and Time. *J. Am. Soc. Mass. Spectrom.* **4**, 524-535.

155. Bruins, A. P. (1998) Mechanistic Aspects of Electrospray Ionization. *J. Chromatogr. A.* **794**, 345-357.

156. Ashton, D. S., Beddell, C. R., Cooper, D. J., Green, B. N., and Oliver, R. W. A. (1993) Mechanism and Production of Ions in Electrospray Mass Spectrometry. *Org. Mass Spectrom.* **28**, 721-728.

157. Bleicher, K., and Bayer, E. (1994) Various Factors Influencing the Signal Intensity of Oligonucleotides in Electrospray Mass-Spectrometry. *Biol. Mass S.* **23**, 320-322.

158. Watkins, N. G., Thorpe, S. R., and Baynes, J. W. (1985) Glycation of Amino Groups in Protein. *J. Biol. Chem.* **260, No. 19**, 10629-10636.

159. Pares, X., Llorens, R., Arus, C., and Cuchillo, C. M. (1980) The reaction of Bovine Pancreatic Ribonuclease A with 6-Chloropurineriboside 5'-Monophosphate. *Eur. J. Biochem.* **105**, 571-579.

160. Alonso, J., Nogues, V., and Cuchillo, C. M. (1986) Modification of Bovine Pancreatic Ribonuclease A with 6-Chloropurine Riboside. *Arch. Biochem. Biophys.* **246**, 681-689.

161. Richards, F. M., and Vithayathil, P. J. (1959) The Preparation of Subtilisin-modified Ribonuclease and the Separation of the Peptide and Protein Components. *J. Biol. Chem.* **234**, 1459-1465.

162. Richards, F. M., and Wyckoff, H. W. (1971) The Enzymes. 3rd edition, 647. New York, Academic Press.

163. Wyckoff, H. W., Tsernoglu, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970) *J. Biol. Chem.* **245**, 305-328.

164. Taylor, H. C., Richardson, D. C., Richardson, J. S., Wlodawer, A., Komoriya, A., and Chaiken, I. M. (1981) Active Conformation of an Inactive Semi-Synthetic Ribonuclease-S. *J. Mol. Biol.* **149**, 313-317.

165. Connelly, P. R., Varadarajan, R., Sturtevant, J. M., and Richards, F. M. (1990) Thermodynamics of Protein-Peptide Interactions in the Ribonuclease S System Studied by Titration Calorimetry. *Biochemistry.* **29**, 6108-6114.

166. Schreier, A. A., and Baldwin, R. L. (1977) Mechanism of Dissociation of S-Peptide from Ribonuclease S. *Biochemistry.* **16**, 4203-4209.

167. Alexandrescu, A. T., Rathgeb-Szabo, K., Rumpel, K., Jahnke, W., Schulthess, T., and Kammerer, R. A. (1998) ^{15}N Backbone Dynamics of the S-Peptide from Ribonuclease A in its Free and S-Protein Bound Forms: Toward a Site-Specific Analysis of Entropy Changes Upon Folding. *Protein Sci.* **7**, 389-402.

168. Ogorzalek Loo, R. R., Goodlett, D. R., Smith, R. D., and Loo, J. A. (1993) Observation of a Noncovalent Ribonuclease S-Protein/S-Peptide Complex by Electrospray Ionization Mass Spectrometry. *J. Am. Chem. Soc.* **115**, 4391-4392.

169. Ihnat, M. (1972) Binding of Copper (II) by Ribonuclease A and Ribonuclease S-Peptide Investigated by H Nuclear Magnetic Resonance Spectroscopy. *Nuclear Magnetic Resonance.* **11**, 3483-3492.

170. Heather, R. W. (1997) LCQ MS Detector Hardware Manual. 5-26.

171. Goodlett, D. R., Ogorzalek Loo, R. R., Loo, J. A., Wahl, J. H., Udseth, H. R., and Smith, R. D. (1994) A Study of the Thermal Denaturation of Ribonuclease S by Electrospray Ionization Mass Spectrometry. *J. Am. Soc. Mass. Spectrom.* **5**, 614-622.

172. Neumann, U., and Hofsteenge, J. (1994) Interaction of Semisynthetic Variants of RNase A with Ribonuclease Inhibitor. *Protein Sci.* **3**, 248-256.

173. Hamachi, I., Hiraoka, T., Yamada, Y., and Shinkai, S. (1998) Photoswitching of the Enzymatic Activity of Semisynthetic Ribonuclease S' Bearing Phenylazophenylalanin at a Specific Site. *Chem. Lett.* 537-538.

174. Zuckermann, R. N., and Schultz, P. G. (1988) A Hybrid Sequence-Selective Ribonuclease S. *J. Am. Chem. Soc.* **110**, 6592-6594.

175. De Rosch, M., and Trogler, W. C. (1990) Hydrolysis of Phosphodiesters with Ni(II), Cu(II), Zn(II), Pd(II), and Pt(II) Complexes. *Inorg. Chem.* **29**, 2409-2416.

176. Chin, J. (1991) Developing Artificial Hydrolytic Metalloenzymes by a Unified Mechanistic Approach. *Acc. Chem. Res.* **24**, 145-152.

177. Anderegg, G. (1971) Pyridinderivate als Komplexbildner IX Die Stabilitatskonstanten von Koplexen mit a) 2-aminomethyl-pyridin, b) 6-Methyl-2-aminomethyl-pyridin, c) 2-Pyridylhydrazin, d) 2,2'-Dipyridylamin und, e) 1-(a-Pyridylmethylen)-2-(a'-pyridyl)-hydrazin. *Helv. Chim. Acta.* **54**, 509.

178. Chin, J., and Jubian, V. (1989) A Highly Efficient Copper(II) Complex Catalysed Hydrolysis of Methyl Acetate at pH 7.0 and 25°C. *J. Chem. Soc., Chem. Commun.* 839-840.

179. Ebright, R. H., Ebright, Y. W., Pendergrast, P. S., and Gunasekera, A. (1990) Conversion of a Helix-Turn-Helix Motif Sequence -Specific DNA Binding Protein into a Site-Specific DNA Cleavage Agent. *Proc. Natl. Acad. Sci. USA.* **87**, 2882-2886.

180. Chen, C. B., Mazumder, A., Constant, J., and Sigman, D. S. (1993) Nuclease Activity of 1, 10-Phenanthroline-Copper. New Conjugates with Low Molecular Weight Targeting Ligands. *Bioconjugate Chem.* **4**, 69-77.

181. Landgraf, R., Pan, C., Sutton, C. L., Pearson, L., and Sigman, D. S. (1996) Engineering of DNA Binding Proteins Into Site-Specific Cutters: Reactivity of Trp Repressor-1,10-Phenanthroline Chimeras. *Protein Engineering.* **9**, 603-610.

182. Sigman, D. S., Mazumder, A., and Perrin, D. M. (1997) Chemical Nucleases. *Chem. Rev.* **93**, 2295-2316.

183. Sigman, D. S., Kuwabara, M. D., Chen, C. B., and Bruice, T. C. (1991) Nuclease Activity of 1,10-Phenanthroline-Copper in Study of Protein-DNA Interactions. **1**, 414-433. New York, Academic Press, Inc.

184. Sigman, D. S., Bruice, T. C., Mazumder, A., and Sutton, C. L. (1993) Targeted Chemical Nucleases. *Acc. Chem. Res.* **26**, 98-104.

185. Hoyer, D., Cho, H., and Schultz, P. G. (1990) A New Strategy for Selective Protein Cleavage. *J. Am. Chem. Soc.* **112**, 3249-3250.

186. Zuckermann, R. N., and Schultz, P. G. (1988) A Hybrid Sequence-Selective Ribonuclease S. *J. Am. Chem. Soc.* **110**, 6592-6594.

187. Mack, D. P., and Dervan, P. B. (1990) Nickel-Mediated Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein. *J. Am. Chem. Soc.* **112**, 4604-4606.

188. Mack, D. P., and Dervan, P. B. (1992) Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein, Ni(II)GGH (Hin 139-190). *Biochemistry.* **31**, 9399-9405.

189. Davies, R. R., and Distefano, M. D. (1997) A Semisynthetic Metalloenzyme Based on a Protein Cavity That Catalyzes the Enantioselective Hydrolysis of Ester and Amide Substrates. *J. Am. Chem. Soc.* **119**, 11643-11652.

190. Schott, H., and Kossel, H. (1973) Synthesis of Phage Specific Deoxyribonucleic Acid Fragments. *J. Am. Chem. Soc.* **95**, 3778.

191. Hutchison, C. A., Phillips, S., Edgell, M. H., Gillam, S., Jahnke, P., and Smith, M. (1978) Mutagenesis at a Specific Position in a DNA-Sequence. *J. Biol. Chem.* **253**, 6551-6560.

192. Shortle, D., Dimaio, D., and Nathans, D. (1981) Directed Mutagenesis. *Annu. Rev. Genet.* **15**, 265-294.

193. Shortle, D. (1987) Genetic Strategies for Analyzing Proteins. Tutorials in Molecular Biology. Fox, C. F., Ed. 103-108. New York, Alan R. Liss.

194. Smith, M. (1985) Invitro Mutagenesis. *Annu. Rev. Genet.* **19**, 423-462.

195. Fersht, A. R., Shi, J. P., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y., and Winter, G. P. (1984) Analysis of Enzyme Structure and Activity by Protein Engineering. *Angew. Chem. Int. Ed. Engl.* **23**, 467-538.

196. Fersht, A. R. (1985) Genetic Engineering and Enzymology: Protein Engineering. **2**, 369-388. New York, W. H. Freeman and Co.

197. Knowles, J. R. (1987) Tinkering with Enzymes - What Are We Learning. *Science.* **236**, 1252-1258.

198. Hutchison, C. A., Swanstrom, R., and Loeb, D. D. (1991) Complete Mutagenesis of Protein Coding Domains. Methods in Enzymology. **202**, 356-390.

199. Soucek, J., Raines, R. T., Haugg, M., Raillard-Yoon, S. A., and Benner, S. A. (1999) Structural Changes to Ribonuclease A and their Effects on Biological Activity. *Comp. Biochem. Physiol. Pt. C.* **123**, 103-111.

200. Ciglic, M. I., Jackson, P. J., Raillard, S. A., Haugg, M., Jermann, T. M., Opitz, J. G., Trabesinger-Ruf, N., and Benner, S. A. (1998) Origin of Dimeric Structure in the Ribonuclease Superfamily. *Biochemistry.* **37**, 4008-4022.

201. Benner, S. A., Jermann, T. M., Opitz, J. G., Raillard, S. A., Zankel, T. R., TrautweinFritz, K., Stackhouse, J., Ciglic, M. I., Haugg, M., TrabesingerRuf, N., and Weinhold, E. G. (1996) Developing New Synthetic Catalysts. How Nature Does It. *Acta Chem. Scand.* **50**, 243-248.

BIOGRAPHICAL SKETCH

Joy was born Trisha Joy Henry, the third child of Bruce and Lora Henry in Newmarket, Ontario, Canada, on September 28, 1970. Joy attended public school from kindergarten through the eighth grade in Barrie, Ontario, Canada. At the age of fourteen she moved with her family to Dallas, Texas, where she entered Lake Highlands Junior High School. She went on to attend and graduate from Lake Highlands High School.

After working for a year following her high school graduation, Joy married Edras Noel Méndez Amaya whom she had met during high school while on a brief trip to Central America. They were married on July 13, 1989, in Dallas, Texas.

Joy then went on to attend college at the University of Texas at Dallas. She did her undergraduate research under the direction of Professor Duane Hrncir studying the bioremediation of metals with constructed wetlands. She earned her Bachelor of Science in chemistry from UTD in the fall of 1994.

One and a half months after her graduation, Joy and Edras became the grateful parents of their son, Edras Alexander Méndez, on February 3, 1995. Joy stayed home to care for her son until she began graduate school in the department of chemistry at the University of Florida in August 1995.

The next summer, Joy began research in the group of her advisor, Professor David E. Richardson. Over the course of her time in graduate school, her research mainly involved bioanalytical chemistry.

Joy's time outside of school is spent on the endeavor she most cherishes, being a wife and mother.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David E. Richardson, Chair
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Benjamin A. Horenstein
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Powell
Scientist of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David N Silverman

David N. Silverman
Professor of Pharmacology and
Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jon D. Stewart
Assistant Professor of Chemistry

This dissertation was submitted to the Graduate Faculty of the Department of Chemistry and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1999

Dean, Graduate School